# CRYSTAL STRUCTURES OF JNK-INHIBITOR COMPLEXES AND BINDING POCKETS THEREOF

## TECHNICAL FIELD OF INVENTION

[0001] The present invention relates to crystalline molecules or molecular complexes that comprise binding pockets of the c-Jun N-terminal kinase 3 (JNK3) and its homologues, the structure of these molecules or molecular complexes, and methods of using these molecules or molecular complexes.

## BACKGROUND OF THE INVENTION

[0002] Mammalian cells respond to extracellular stimuli by activating signaling cascades that are mediated by members of the mitogen-activated protein (MAP) kinase family, which include the extracellular signal regulated kinases (ERKs), the p38 MAP kinases and the c-Jun N-terminal kinases (JNK). MAP kinases are serine/threonine kinases that are activated by dual phosphorylation of threonine and tyrosine at the Thr-X-Tyr segment in the activation loop. MAP kinases phosphorylate various substrates including transcription factors, which in turn regulate the expression of specific sets of genes and thus mediate a specific response to a specific stimulus.

[0003] Three distinct genes, JNK1, JNK2, JNK3 have

been identified and at least ten different splicing isoforms of JNK exist in mammalian cells [S. Gupta et al., EMBO J., 15, pp. 2760-2770 (1996)]. Members of the JNK kinases are activated by proinflammatory cytokines tumor necrosis factor-alpha and interleukin-1 beta as well as environmental stress, such as anisomycin, UV irradiation, hypoxia, and osmotic shock [A. Minden et al., Biochemica et Biophysica Acta, 1333, F85-F104 (1997)]. The down-stream substrates of JNK proteins include transcription factors c-Jun, ATF-2, Elk1, p53 and a cell death domain protein (DENN) [Y. Zhang et al. Proc. Natl. Acad. Sci. USA, 95, pp. 2586-2591 (1998)]. Each of the JNK isoforms binds to these substrates with a different affinity, suggesting a regulation of signaling pathways by substrate specificity of different JNK proteins *in vivo* [S. Gupta et al., 1996].

**[0004]** JNK1 and JNK2 are widely expressed in a variety of tissues. In contrast, JNK3 is selectively expressed in the brain and to a lesser extent in the heart and testis [S. Gupta et al., (1996), *supra*; A. A. Mohit et al., Neuron, 14, pp. 67-78 (1995); J.H. Martin et al., Brain Res. Mol. Brain Res., 35, pp. 47-57 (1996)]. In the adult human brain, JNK3 expression is localized to a subpopulation of pyramidal neurons in the CA1, CA4 and subiculum regions of the hippocampus and layers 3 and 5 of the neocortex [A. A. Mohit et al. (1995), *supra*]. The CA1 neurons of patients with acute hypoxia showed strong nuclear JNK3-immunoreactivity compared to minimal, diffuse cytoplasmic staining of the hippocampal neurons from brain tissues of normal patients [Y. Zhang et al. (1998), *supra*]. In addition, JNK3 co-localizes immunochemically with neurons

vulnerable in Alzheimer's disease [A. A. Mohit et al., (1995), *supra*]. Disruption of the JNK3 gene caused resistance of mice to the excitotoxic glutamate receptor agonist kainic acid, which affected seizure activity, AP-1 transcriptional activity and apoptosis of hippocampal neurons, indicating that the JNK3 signaling pathway is a critical component in the pathogenesis of glutamate neurotoxicity (D. D. Yang et al., Nature, 389, pp. 865-870 (1997)]. Thus, selective modulation of JNK3 activity could potentially provide therapeutic intervention for neurodegenerative diseases such as stroke and epilepsy.

**[0005]** Accordingly, there has been an interest in identifying JNK3 inhibitors that are effective as therapeutic agents. A challenge has been to provide protein kinase inhibitors that act in a selective manner. Since there are numerous protein kinases that are involved in a variety of cellular responses, non-selective inhibitors may lead to unwanted side effects.

**[0006]** Further, the binding of different inhibitors may alter the conformation of the binding pocket. Information provided by the X-ray crystal structure of JNK3-inhibitor complexes would be extremely useful in iterative drug design of various JNK proteins. The determination of the amino acid residues in JNK3 binding pockets and the determination of the shape of those binding pockets would allow one to modify inhibitors to bind more favorably to this class of enzymes.

## SUMMARY OF THE INVENTION

[0007] Applicants have solved these problems by providing a crystal comprising JNK3 or homologue thereof in complex with an inhibitor. Solving these crystal structures has allowed applicants to determine the shape of the inhibitor-binding pocket and amino acid residues in the pocket. The invention also provides a method for crystallizing JNK-inhibitor complexes.

[0008] Thus, the present invention provides crystalline molecules or molecular complexes comprising the JNK3 inhibitor-binding pockets, or JNK3-like inhibitor-binding pockets that have similar three-dimensional shapes.

[0009] The invention further provides a computer comprising a data storage medium that comprises the structure coordinates of molecules and molecular complexes comprising all or part of the JNK3 or JNK3-like binding pockets and means for generating three-dimensional structural information from the structure coordinates. The computer may be used to produce three-dimensional information of the crystalline molecule or molecular complex comprising such binding pockets.

[0010] The invention provides methods for screening, designing, optimizing, evaluating and identifying compounds that bind to the molecules or molecular complexes or their binding pockets. The methods can be used to identify agonists and antagonists of JNK3 and its homologues.

[0011] The invention also provides a method for determining at least a portion of the three-dimensional structure of molecules or molecular complexes which

contain at least some structurally similar features to JNK3, particularly JNK3 homologues. This is achieved by using at least some of the structural coordinates obtained from the JNK3-inhibitor structures.

## BRIEF DESCRIPTION OF THE FIGURES

**[0012]** Figure 1 lists the atomic structure coordinates for unphosphorylated JNK3 in complex with N-[4-(5-Methyl-3-phenyl-isoxazol-4-yl)-pyrimidin-2-yl]-acetamide [see WO 200112621, incorporated herein by reference] (hereinafter "isoxazole1"), as derived by X-ray diffraction from a crystal of that complex.

**[0013]** Figure 2 lists the atomic structure coordinates for unphosphorylated JNK3 in complex with 2,4-Dioxo-6-phenylamino-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid phenylamide [see WO 2000075118, incorporated herein by reference] (hereinafter "uracil1") as derived by X-ray diffraction from a crystal of that complex.

**[0014]** Figure 3 lists the atomic structure coordinates for unphosphorylated JNK3 in complex with 2-Pyridin-4-yl-thiazole-4-carboxylic acid(3-trifluoromethyl-phenyl)-amide] [see U.S. patent 6,274,738, incorporated herein by reference] (herein after "thiazole1") as derived by X-ray diffraction from a crystal of that complex.

**[0015]** The following abbreviations are used in Figure 1, 2 and 3:

**[0016]** "Atom type" refers to the element whose coordinates are measured. The first letter in the column defines the element.

**[0017]** "Res" refers to the amino acid residue in the molecular model.

[0018] "X, Y, Z" define the atomic position of the element measured.

[0019] "B" is a thermal factor that measures movement of the atom around its atomic center.

[0020] "Occ" is an occupancy factor that refers to the fraction of the molecules in which each atom occupies the position specified by the coordinates. A value of "1" indicates that each atom has the same conformation, i.e., the same position, in all molecules of the crystal.

[0021] Figure 4 is a structure-based sequence alignment of JNK3, ERK2, p38 and cAPK (SEQ ID NO: 1, 2, 3 and 4, respectively).

[0022] Figure 5 is a ribbon representation of the overall fold of JNK3. The N-terminal $\beta$ strands and C-terminal $\alpha$ helices are shown.

[0023] Figure 6 shows a detailed comparison of the active site of JNK3-uracil1 with that of JNK3-AMP-PNP. The C$\alpha$ of residues 140-149 of the two structures were superimposed. The figure illustrates the protein conformational changes induced by inhibitor binding. Figure 6A is viewed from the entrance of the cleft where ATP binds; Figure 6B is viewed perpendicular to 6A. Certain distances between atoms are indicated by dashed lines.

[0024] Figure 7 depicts an overlay of the structures of AMP-PNP, uracil1, thiazole1 and isoxazole1.

[0025] Figure 8 shows the three inhibitors bound in the inhibitor-binding pocket. Figure 8A (thiazole1), Figure 8B (uracil1), Figure 8C (isoxazole1). Hydrogen bonds are indicated by dashed lines.

[0026] Figure 9 shows a diagram of a system used to carry out the instructions encoded by the storage medium of Figures 10 and 11.

[0027] Figure 10 shows a cross section of a magnetic storage medium.

[0028] Figure 11 shows a cross section of a optically-readable data storage medium.

## DETAILED DESCRIPTION OF THE INVENTION

[0029] In order that the invention described herein may be more fully understood, the following detailed description is set forth.

[0030] Throughout the specification, the word "comprise", or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or groups of integers but not exclusion of any other integer or groups of integers.

[0031] The following abbreviations are used throughout the application:

| | |
|---|---|
| A = Ala = Alanine | T = Thr = Threonine |
| V = Val = Valine | C = Cys = Cysteine |
| L = Leu = Leucine | Y = Tyr = Tyrosine |
| I = Ile = Isoleucine | N = Asn = Asparagine |
| P = Pro = Proline | Q = Gln = Glutamine |
| F = Phe = Phenylalanine | D = Asp = Aspartic Acid |
| W = Trp = Tryptophan | E = Glu = Glutamic Acid |
| M = Met = Methionine | K = Lys = Lysine |
| G = Gly = Glycine | R = Arg = Arginine |
| S = Ser = Serine | H = His = Histidine |

[0032] The term "about" when used in the context of RMSD values takes into consideration the standard error of the RMSD value, which is ± 0.1 Å.

[0033] The term "active site" refers to the portion of the protein kinase to which the nucleotide substrate binds. This site is located at the interface of the N-terminal α-helical and C-terminal β-strand domain, and is bordered by the glycine rich loop and the hinge [See, Xie et al., Structure, 6, pp. 983-991 (1998), incorporated herein by reference].

[0034] The term "associating with" refers to a condition of proximity between a chemical entity or compound, or portions thereof, and a binding pocket or binding site on a protein. The association may be non-covalent -- wherein the juxtaposition is energetically favored by hydrogen bonding or van der Waals or electrostatic interactions -- or it may be covalent.

[0035] The term "binding pocket" refers to a region of a molecule or molecular complex, that, as a result of its shape, favorably associates with another chemical entity or compound.

[0036] The term "chemical entity" refers to chemical compounds, complexes of at least two chemical compounds, and fragments of such compounds or complexes. The chemical entity may be, for example, a ligand, a substrate, a nucleotide triphosphate, a nucleotide diphosphate, phosphate, a nucleotide, an agonist, antagonist, inhibitor, antibody, drug, peptide, protein or compound.

[0037] The term "conservative substitutions" refers to residues that are physically or functionally similar to the corresponding reference residues. That is, a conservative substitution and its reference residue

have similar size, shape, electric charge, chemical properties including the ability to form covalent or hydrogen bonds, or the like. Preferred conservative substitutions are those fulfilling the criteria defined for an accepted point mutation in Dayhoff *et al.*, Atlas of Protein Sequence and Structure, 5, pp. 345-352 (1978 & Supp.), which is incorporated herein by reference. Examples of conservative substitutions are substitutions including but not limited to the following groups: (a) valine, glycine; (b) glycine, alanine; (c) valine, isoleucine, leucine; (d) aspartic acid, glutamic acid; (e) asparagine, glutamine; (f) serine, threonine; (g) lysine, arginine, methionine; and (h) phenylalanine, tyrosine.

**[0038]** The term "corresponding amino acid" or "residue which corresponds to" refers to a particular amino acid or analogue thereof in a JNK3 protein or JNK3 homologue that corresponds to an amino acid in JNK3α1. The corresponding amino acid is identical or functionally equivalent to the JNK3α1 amino acid to which it corresponds.

**[0039]** Methods for identifying a corresponding amino acid are known in the art and are based upon sequence, structural alignment, its functional position or a combination thereof as compared to the JNK3 kinase. For example, corresponding amino acids may be identified by superimposing the backbone atoms of the amino acids in JNK3 and the JNK3 homologue using well known software applications, such as QUANTA (Accelrys, San Diego, CA ©2001, 2002). The corresponding amino acids may also be identified using sequence alignment programs such as the "bestfit" program available from the Genetics Computer Group which uses the local

homology algorithm described by Smith and Waterman in Advances in Applied Mathematics 2, 482 (1981), which is incorporated herein by reference.

**[0040]** The term "crystallization solution" refers to a solution that promotes crystallization. The solution comprises at least one agent, and may include a buffer, one or more salts, a precipitating agent, one or more detergents, sugars or organic compounds, lanthanide ions, a poly-ionic compound and/or a stabilizer.

**[0041]** The term "domain" refers to a structural unit of the JNK3 protein or homologue. The domain can comprise a binding pocket, or a sequence or structural motif. In JNK3, the protein is separated into two domains, the N-terminal domain which contains $\beta$ strands and the C-terminal domain which is predominantly $\alpha$ helical.

**[0042]** The term "generating a three-dimensional structure" refers to converting the lists of structure coordinates into structural models in three-dimensional space. This can be achieved through commercially or publicly available software. The three-dimensional structure may be displayed as a graphical representation or used to perform computer modeling or fitting operations. In addition, the structure coordinates themselves may be used to perform computer modeling and fitting operations.

**[0043]** The term "homology model" refers to a structural model derived from known three-dimensional structure(s). Generation of the homology model, termed "homology modeling", can include sequence alignment, residue replacement, residue conformation adjustment through energy minimization, or a combination thereof

[0044] The term "homologue of JNK3" or "JNK3 homologue" refers to a molecule that is homologous to JNK3 by three-dimensional structure or sequence and retains the kinase activity of a JNK protein. Examples of homologues include but are not limited to the following: JNK protein such as JNK1, JNK2 and JNK3 with conservative substitutions, additions, deletions or a combination thereof; other JNK proteins such as JNK1, JNK2 or isoforms thereof.

[0045] The term "JNK" refers to the kinases from the c-Jun N-terminal kinase family. Examples of this family of kinases include but are not limited to JNK1, JNK2, JNK3 and isoforms thereof. Isoforms of JNK1, JNK2 and JNK3 include but are not limited to JNK1α1, JNK1α2, JNK1β1, JNK1β2, JNK2α1, JNK2α2, JNK2β1, JNK2β2, JNK3α1, JNK3α2, JNK3β1, JNK3β2, respectively.

[0046] The term "JNK3 inhibitor-binding pocket" refers to that portion of the JNK3 enzyme active site to which the inhibitor binds. The inhibitor-binding pocket is defined by the structure coordinates of a certain set of amino acid residues present in the JNK3-inhibitor structure, as described below. The amino acid residues and the shape of the inhibitor-binding pocket according to this invention differ from those of the active site binding pocket described in the JNK3-AMP-PNP structure [WO 9957253].

[0047] The term "JNK3-like" refers to all or a portion of a molecule or molecular complex that has a commonality of shape to all or a portion of the JNK3 protein. In the JNK3-like inhibitor-binding pocket, the commonality of shape is defined by a root mean square deviation of the structure coordinates of the backbone atoms between the amino acids in the JNK3-like

inhibitor-binding pocket and the amino acids in the JNK3 inhibitor-binding pocket (as set forth in Figure 1 2 or 3).

**[0048]** The term "part of a JNK3 inhibitor-binding pocket" or "part of a JNK3-like inhibitor-binding pocket" refers to less than all of the amino acid residues that define the JNK3 or JNK3-like inhibitor-binding pocket. The structure coordinates of residues that constitute part of a JNK3 or JNK3-like inhibitor-binding pocket may be specific for defining the chemical environment of the binding pocket, or useful in designing fragments of an inhibitor that may interact with those residues. For example, the portion of residues may be key residues that play a role in ligand binding, or may be residues that are spatially related and define a three-dimensional compartment of the binding pocket. The residues may be contiguous or non-contiguous in primary sequence.

**[0049]** In one embodiment, part of the JNK3 or JNK3-like inhibitor-binding pocket is at least two amino acid residues. Preferably, the amino acids are Lys93 and Met146. In one embodiment, part of an inhibitor-binding pocket, comprises at least one residue that is not found within 5 or 8 Å of the AMP-PNP in the JNK3-AMP-PNP structure [WO 9957253]. Examples of these residues include Pro69, Met115, Leu126, Leu127, Asn128, Tyr143, Leu144, Val145, Lys198 and Ile205. In another embodiment, part of an inhibitor-binding pocket comprises at least one residue that underwent conformational change when compared to the residues in the JNK3-AMP-PNP structure. Examples of these residues include but are not limited to Lys93, Ile124, Leu126, Leu144 and Met146. In another embodiment, part of an

inhibitor-binding pocket comprises Ile70, Gly71, Ser72, Asn152, Cys154 and Gln155. In another embodiment, part of any inhibitor-binding pocket comprises Ser72, Ser193 and Asn152.

**[0050]** The term "part of a JNK3 protein" or "part of a JNK3 homologue" refers to less than all of the amino acid residues of a JNK3 protein or homologue. In one embodiment, part of a JNK3 protein or homologue defines the binding pockets, domains or motifs of the protein or homologue. The structure coordinates of residues that constitute part of a JNK3 protein or homologue may be specific for defining the chemical environment of the protein, or useful in designing fragments of an inhibitor that may interact with those residues. The portion of residues may also be residues that are spatially related and define a three-dimensional compartment of a binding pocket, motif or domain. The residues may be contiguous or non-contiguous in primary sequence. For example, the portion of residues may be key residues that play a role in ligand or substrate binding, catalysis or structural stabilization.

**[0051]** The term "protein complex" refers to a protein associated with a chemical entity, for example, a ligand, a substrate, nucleotide triphosphate, an agonist, an antagonist, inhibitor, drug or compound. In one embodiment, the chemical entity is an inhibitor that induces the Met146 in JNK3 or corresponding methionine in a JNK3 homologue to have a $\chi 1$ angle in the range of -120° to -180° and 45° to 180° upon binding. In one embodiment, the chemical entity is selected from the group consisting of N-[4-(5-Methyl-3-phenyl-isoxazol-4-yl)-pyrimidin-2-yl]-acetamide, 2,4-Dioxo-6-phenylamino-1,2,3,4-tetrahydro-pyrimidine-5-

carboxylic acid phenylamide, 2-Pyridin-4-yl-thiazole-4-carboxylic acid(3-trifluoromethyl-phenyl)-amide, 4-[5-(4-Fluoro-phenyl)-4-pyridin-4-yl-1*H*-imidazol-2-yl]-phenol and 2-(2,6-Dichloro-phenyl)-2-[5-(2,4-difluorobenzoyl)-pyridin-2-yl]-acetamide.

**[0052]** The term "motif" refers to a portion of the JNK3 kinase or homologue that defines a structural compartment or carries out a function in the protein, for example, catalysis, structural stabilization or phosphorylation. The motif may be conserved in sequence, structure and function when compared to other kinases or related proteins. The motif can be contiguous in primary sequence or three-dimensional space. The motif may comprise $\alpha$-helices and $\beta$-sheets. Examples of a motif include but are not limited to a binding pocket, active site, phosphorylation lip or activation loop, the glycine-rich phosphate anchor loop, the catalytic loop and the DFG loop [See, Xie et al., Structure, 6, pp. 983-991 (1998)].

**[0053]** The term "root mean square deviation" or "RMSD" means the square root of the arithmetic mean of the squares of the deviations from the mean. It is a way to express the deviation or variation from a trend or object. The "root mean square deviation" may define the variation in the backbone and/or sidechains of a protein from the backbone and/or sidechains of JNK3, a binding pocket, a motif, a domain, or portion thereof, as defined by the structure coordinates of JNK3 described herein.

**[0054]** The term "soaked" refers to a process in which the crystal is transferred to a solution containing the compound to be diffused into the crystal.

[0055] The term "structure coordinates" refers to Cartesian coordinates derived from mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of X-rays by the atoms (scattering centers) of a protein or protein complex in crystal form. The diffraction data are used to calculate an electron density map of the repeating unit of the crystal. The electron density maps are then used to establish the positions of the individual atoms of the molecule or molecular complex.

[0056] The term "sufficiently homologous to JNK3" refers to a protein that has a sequence homology of at least 20% compared to JNK3 protein. In other embodiments, the sequence homology is at least 40%, at least 60%, at least 80%, at least 90% or at least 95%.

[0057] The term "three-dimensional structural information" refers to information obtained from the structure coordinates. Structural information generated can include the three-dimensional structure or graphical representation of the structure. Structural information can also be generated when subtracting distances between atoms in the structure coordinates, calculating chemical energies for a JNK3 molecule or molecular complex or homologues thereof, calculating or minimizing energies for an association of a JNK3 molecule or molecular complex or homologues thereof to a chemical entity.

Crystallizable Compositions and Crystals of JNK3-inhibitor Complexes

[0058] According to one embodiment, the invention provides a crystallizable composition or crystal comprising a JNK3 protein complexed with an inhibitor

or JNK3 homologue complexed with an inhibitor. In one embodiment, the JNK3 protein or homologue is phosphorylated or unphosphorylated. In another embodiment, the JNK3 protein is JNK3α1. In one embodiment, the inhibitor is capable of inducing the Met146 in JNK3 or corresponding methionine in a JNK3 homologue to have a χ1 angle in the range of -120° to -180° and 45° to 180° upon binding. In one embodiment, the inhibitor is selected from the group consisting of N-[4-(5-Methyl-3-phenyl-isoxazol-4-yl)-pyrimidin-2-yl]-acetamide, 2,4-Dioxo-6-phenylamino-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid phenylamide, 2-Pyridin-4-yl-thiazole-4-carboxylic acid(3-trifluoromethyl-phenyl)-amide, 4-[5-(4-Fluoro-phenyl)-4-pyridin-4-yl-1*H*-imidazol-2-yl]-phenol and 2-(2,6-Dichloro-phenyl)-2-[5-(2,4-difluorobenzoyl)-pyridin-2-yl]-acetamide.

**[0059]** For JNK3 protein or a JNK3 protein with amino acid substitutions, the N-terminus of the protein can be truncated. Specifically, the JNK3 proteins contain an N-terminal extension of about 40 amino acids as compared to JNK1 and JNK2 proteins (see for example, GenBank entries for JNK1, JNK2 and JNK3 proteins, NP620637, S71102 and S71104, respectively). In one embodiment, those 40 amino acids are removed from JNK3 proteins in the crystallizable or crystal compositions of this invention.

**[0060]** In addition, any JNK3 protein or homologue thereof in these crystal or crystallizable compositions preferably has a C-terminal truncation of about 20 amino acids. The C-terminal truncation is helpful in obtaining diffraction quality crystals.

**[0061]** The crystallizable compositions may further comprise a crystallization solution of polyethylene

glycol monomethyl ether at between about 10 to 30% v/v, ethylene glycol at between about 5 to 20% v/v, a reducing agent, such as ß-mercaptoethanol at between about 5 to 50 mM, and a buffer that maintains pH at between about 7.0 and 7.5. Preferably, the buffer is 100 mM Hepes at pH 7.0.

**[0062]** In one embodiment, the crystal has a unit cell dimension of a= 54.75 Å b= 70.48 Å, c= 107.66 Å, $\alpha = \beta = \gamma = 90°$ and belongs to space group *P212121*. In another embodiment, the crystal has a unit cell dimension of a= 51.65 Å b= 71.01 Å, c= 106.7 Å, $\alpha = \beta = \gamma = 90°$ and belongs to space group *P212121*. In another embodiment, the crystal has a unit cell dimension of a= 50.74 Å b= 71.82 Å, c= 107.22 Å, $\alpha = \beta = \gamma = 90°$ and belongs to space group *P212121*. It will be readily apparent to those skilled in the art that the unit cells of the crystal compositions may deviate upto ± 1-2 Å from the above cell dimensions depending on the deviation in the unit cell calculations or conformational change in the protein.

**[0063]** The JNK3 protein or homologue thereof may be produced by any well-known method, including synthetic methods, such as solid phase, liquid phase and combination solid phase/liquid phase syntheses; recombinant DNA methods, including cDNA cloning, optionally combined with site directed mutagenesis; and/or purification of the natural products. In one embodiment, the protein is overexpressed from an *E. coli* system.

**[0064]** The invention also relates to a method of making crystals of JNK3-inhibitor complexes or JNK3 homologue-inhibitor complexes. Such methods comprise the steps of:

a) producing a composition comprising a crystallization solution and a JNK3 protein or homologue thereof complexed with an inhibitor; and

b) subjecting said composition to devices or conditions which promote crystallization.

**[0065]** In one embodiment, the inhibitor is capable of inducing the Met146 in JNK3 or corresponding methionine in the JNK3 homologue to have a $\chi 1$ angle in the range of -120° to -180° and 45° to 180° upon binding. In another embodiment, the inhibitor is selected from the group consisting of N-[4-(5-Methyl-3-phenyl-isoxazol-4-yl)-pyrimidin-2-yl]-acetamide, 2,4-Dioxo-6-phenylamino-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid phenylamide, 2-Pyridin-4-yl-thiazole-4-carboxylic acid(3-trifluoromethyl-phenyl)-amide, 4-[5-(4-Fluoro-phenyl)-4-pyridin-4-yl-1*H*-imidazol-2-yl]-phenol and 2-(2,6-Dichloro-phenyl)-2-[5-(2,4-difluorobenzoyl)-pyridin-2-yl]-acetamide. In another embodiment, the crystallization solution is as described previously. In another embodiment, the composition is treated with micro-crystals of JNK3 or JNK3 complexes or homologues thereof after step (a) but prior to step (b).

**[0066]** In each of the above embodiments, it is preferred that the JNK3 protein is JNK3$\alpha$1.

**[0067]** Devices for promoting crystallization can include but are not limited to the hanging-drop, sitting-drop, dialysis or microtube batch devices. [U.S. patent 4,886,646, 5,096,676, 5,130,105, 5,221,410 and 5,400,741; Pav *et al.*, Proteins: Structure, Function, and Genetics, 20, pp. 98-102 (1994), incorporated herein by reference]. The hanging-drop or sitting-drop methods produce crystals by vapor

diffusion. The hanging-drop, sitting-drop, and some adaptations of the microbatch methods [D'Arcy et al., J. Cryst. Growth, 168, pp. 175-180 (1996) and Chayen, J. Appl. Cryst., 30, pp. 198-202 (1997)] produce crystals by vapor diffusion. The hanging drop and sitting drop containing the crystallizable composition is equilibrated in a reservoir containing a higher or lower concentration of the precipitant. As the drop approaches equilibrium with the reservoir, the saturation of protein in the solution leads to the formation of crystals.

**[0068]** Microseeding or seeding may be used to obtain larger, or better quality (i.e., crystals with higher resolution diffraction or single crystals) crystals from initial micro-crystals. Microseeding involves the use of crystalline particles to provide nucleation under controlled crystallization conditions. Microseeding is used to increase the size and quality of crystals. In this instance, micro-crystals are crushed to yield a stock seed solution. The stock seed solution is diluted in series. Using a needle, glass rod or strand of hair, a small sample from each diluted solution is added to a set of equilibrated drops containing a protein concentration equal to or less than a concentration needed to create crystals without the presence of seeds. The aim is to end up with a single seed crystal that will act to nucleate crystal growth in the drop.

**[0069]** It would be readily apparent to one of skill in the art following the teachings of the specification to vary the crystallization conditions disclosed herein to identify other crystallization conditions that would produce crystals of JNK3 homologue, JNK3 homologue

complex, other JNK3 proteins or JNK3 protein complexes. Such variations include, but are not limited to, adjusting pH, protein concentration and/or crystallization temperature, changing the identity or concentration of salt and/or precipitant used, using a different method of crystallization, or introducing additives such as detergents (e.g., TWEEN 20 (monolaurate), LDAO, Brij 30 (4 lauryl ether)), sugars (e.g., glucose, maltose), organic compounds (e.g., dioxane, dimethylformamide), lanthanide ions or polyionic compounds that aid in crystallization. High throughput crystallization assays may also be used to assist in finding or optimizing the crystallization conditions.

Binding Pockets of JNK3 Protein or Homologues thereof

**[0070]** As mentioned above, applicants have solved the three-dimensional X-ray crystal structure of JNK3α1 in complex with inhibitors. The atomic coordinate data is presented in Figures 1, 2 and 3.

**[0071]** In order to use the structure coordinates generated for the JNK3-inhibitor complex or one of its binding pockets or homologues thereof, it is often times necessary to convert the structure coordinates into a three-dimensional shape. This is achieved through the use of commercially available software that is capable of generating the three-dimensional structure of molecules or portions thereof from a set of structure coordinates.

**[0072]** Binding pockets, also referred to as binding sites in the present invention, are of significant utility in fields such as drug discovery. The association of natural ligands or substrates with the

binding pockets of their corresponding receptors or enzymes is the basis of many biological mechanisms of action. Similarly, many drugs exert their biological effects through association with the binding pockets of receptors and enzymes. An understanding of such associations will help lead to the design of drugs having more favorable associations with their target receptor or enzyme, and thus, improved biological effects. Therefore, this information is valuable in designing potential inhibitors of the binding sites of biologically important targets.

**[0073]** The structure coordinates described above may be used to derive the torsion angles of the side chains [S.C. Lovell et al, Proteins: Structure, Function, and Genetics, 40, 389-408, (2000)]. For example, in methionine, $\chi 1$ defines the torsion angle between N, $C\alpha$, $C\beta$, $S\gamma$; $\chi 2$ defines the torsion angle between $C\alpha$, $C\beta$, $S\gamma$, $C\delta$; and $\chi 3$ defines the torsion angle between $C\beta$, $S\gamma$, $C\delta$, $C\epsilon$.

**[0074]** Surprisingly, it has now been found that for the JNK3-isoxazole1 and JNK3-uracil1 complexes (Figure 1 and 2), the conformation of Met146 is very different from the conformations reported for methionines at this position in JNK3-AMP-PNP and other protein kinases. A novel hydrophobic pocket is formed around Met146 upon binding of isoxazole1 and uracil1. In order to compare the conformations of JNK3 and other protein kinases at a particular amino acid site, such as Met146, along the polypeptide backbone, well-known procedures may be used for performing sequence alignments of the amino acids. Such sequence alignments allow for the equivalent sites to be compared (see Figure 4). One such method for

performing a sequence alignment is the "bestfit" program available from Genetics Computer Group which uses the local homology algorithm described by Smith and Waterman in Advances in Applied Mathematics 2, 482 (1981).

**[0075]** A suitable amino acid sequence alignment will require that the proteins being aligned share a minimum percentage of identical amino acids. Generally, a first protein being aligned with a second protein should share in excess of about 35% identical amino acids. Hanks et al., Science, 241, 42 (1988); Hanks and Quinn, Methods in Enzymology, 200, 38 (1991).

**[0076]** Equivalents of the Met146 residue of JNK3 may also be identified by its functional position. Met146 is located in the beginning of the hinge region, and is adjacent to residue Glu147. Glu147 uses its backbone carbonyl to form a hydrogen bond with the amino group (N6) of the adenine base from AMP-PNP in the JNK3-AMP-PNP structure [Xie et al., Structure, 6, pp. 983-991 (1998)]. A comparison of the torsion angles between Met146 in the JNK3-inhibitor complexes and those of corresponding methionines in other kinases are illustrated in Table 1. The torsion angles were determined by the program QUANTA.

Table 1

| Proteins | χ1 (°) | χ2 (°) | χ3 (°) |
|---|---|---|---|
| JNK3-isoxazole1 | -159 | -162 | 106 |
| JNK3-uracil1 | 72 | 165 | -55 |
| JNK3-thiazole1 | -54 | 128 | -59 |
| JNK3-AMP-PNP | -35 | 154 | -86 |
| P38γ-AMP-PNP [a] (Met109) | -55 | 107 | 56 |
| JNK3-compound1 [b] | -173 | -164 | 125 |
| JNK3-compound2 [c] | 144 | -127 | -164 |

a Bellon et al., Structure Fold Des., 7, pp.1057 (1999); PDB accession number 1CM8.

b Compound1 is 4-[5-(4-Fluoro-phenyl)-4-pyridin-4-yl-1*H*-imidazol-2-yl]-phenol [see United States Patent 5,916,891, incorporated herein by reference.]

c Compound2 is 2-(2,6-Dichloro-phenyl)-2-[5-(2,4-difluorobenzoyl)-pyridin-2-yl]-acetamide [see United States Patent 6,147,080, incorporated herein by reference].

**[0077]** In addition, applicants have determined that JNK3 amino acids Ile70, Ser72, Val78, Ala91, Ile92, Lys93, Ile124, Leu126, Leu144, Val145, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Ser193, Val196 and Leu206 are within 5 Å of isoxazole1. These amino acids were identified using the program CNX (Accelrys, ©2001). Thus, a binding pocket defined by the structural coordinates of these amino acids, as set forth in Figure 1 is considered a JNK3-inhibitor

binding pocket of this invention. These amino acids differ from the amino acids that are within 5 Å of the AMP-PNP in the JNK3-AMP-PNP structure [WO 9957253]. For example, in the JNK3-AMP-PNP structure, Leu126, Leu144 and Val145 were not found to be within 5 Å of the AMP-PNP.

**[0078]** Applicants have determined that in addition to the above amino acids, JNK3 amino acids Pro69, Gly71, Gln75, Gly76, Ile77, Cys79, Ala80, Val90, Lys94, Leu95, Met115, Ser125, Leu127, Asn128, Tyr143, Leu153, Gln155, Pro192, Asn194, Ile195, Val197, Lys204 and Asp207 are within 8 Å of isoxazole1. These amino acids were identified using the program CNX (Accelrys, ©2001) Thus, a binding pocket defined by the structural coordinates of these amino acids, as set forth in Figure 1 is considered a JNK3-inhibitor binding pocket of this invention. These amino acids differ from the amino acids that are within 8 Å of the AMP-PNP in the JNK3-AMP-PNP structure [WO 9957253]. For example, in the JNK3-AMP-PNP structure, Pro69, Met115, Leu126, Leu127, Asn128, Tyr143 were not found to be within 8 Å of the AMP-PNP.

**[0079]** Applicants have also determined that JNK3 amino acids Ile70, Gln75, Val78, Ala91, Ile92, Lys93, Ile124, Leu126, Leu144, Val145, Met146, Leu148, Met149, Asp150, Ala151, Asn152, Ser193, Asn194, Val196 and Leu206 are within 5 Å of uracil1. These amino acids were identified using the program CNX (Accelrys, ©2001). Thus, a binding pocket defined by the structural coordinates of these amino acids, as set forth in Figure 2 is considered a JNK3-inhibitor binding pocket of this invention. These amino acids differ from the amino acids that are within 5 Å of the

AMP-PNP in the JNK3-AMP-PNP structure [WO 9957253]. For example, in the JNK3-AMP-PNP structure, Leu126, Leu144 and Val145 were not found to be within 5 Å of the AMP-PNP.

**[0080]** Applicants have determined that in addition to the above amino acids, JNK3 amino acids Gly73, Gly76, Ile77, Cys79, Ala80, Val90, Lys94, Leu95, Arg107, Glu111, Met115, Ser125, Leu127, Asn128, Tyr143, Glu147, Leu153, Cys154, Gln155, Pro192, Ile195, Val197, Ile205 and Asp207 are within 8 Å of uracil1. These amino acids were identified using the program CNX (Accelrys, ©2001). Thus, a binding pocket defined by the structural coordinates of these amino acids, as set forth in Figure 2 is considered a JNK3-inhibitor binding pocket of this invention. These amino acids differ from the amino acids that are within 8 Å of the AMP-PNP in the JNK3-AMP-PNP structure [WO 9957253]. For example, in the JNK3-AMP-PNP structure, Met115, Leu126, Leu127, Asn128, Tyr143, Ile205 were not found to be within 8 Å of the AMP-PNP.

**[0081]** In the above inhibitor-binding pockets (Figure 1 or 2), Leu144, Met146, Ile124, Leu126 and Lys93 show significant variation in conformation from those residues in the JNK3-AMP-PNP structure [WO 9957253].

**[0082]** Applicants have also determined that JNK3 amino acids Ile70, Val78, Ala91, Lys93, Glu111, Ile124, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Gln155, Val196 and Leu206 are within 5 Å of thiazole1. These amino acids were identified using the program CNX (Accelrys, ©2001). Thus, a binding pocket defined by the structural coordinates of those amino acids, as set forth in Figure 3 is considered a JNK3-inhibitor

binding pocket of this invention. Compared to these amino acids, there are more amino acids that are within 5 Å of the AMP-PNP in the JNK3-AMP-PNP structure [WO 9957253].

**[0083]** Applicants have determined that in addition to the above amino acids, JNK3 amino acids Gly71, Ser72, Ile77, Cys79, Ala80, Val90, Ile92, Lys94, Leu95, Met115, Ser125, Leu144, Val145, Leu153, Cys154, Ser193, Asn194, Ile195, Val197, Lys198, Lys204, Ile205 and Asp207 are within 8 Å of thiazole1. These amino acids were identified using the program CNX (Accelrys, ©2001). Thus, a binding pocket defined by the structural coordinates of those amino acids, as set forth in Figure 3 is considered a JNK3-inhibitor binding pocket of this invention. These amino acids differ from the amino acids that are within 8 Å of the AMP-PNP in the JNK3-AMP-PNP structure [WO 9957253]. For example, in the JNK3-AMP-PNP structure, Met115, Lys198, Ile205 were not found to be within 8 Å of the AMP-PNP.

**[0084]** In addition, applicants have identified an inhibitor-binding pocket in the JNK3-thiazole1 structure that regulates the binding affinity of inhibitors. This inhibitor-binding pocket is defined by JNK3 amino acids Ile70, Gly71, Ser72, Asn152, Cys154 and Gln155 according to Figure 3.

**[0085]** Applicants have also identified an inhibitor-binding pocket in the JNK3-uracil1 structure that may regulate the binding affinity of inhibitors. This inhibitor-binding pocket is defined by JNK3 amino acids Ser72, Ser193 and Asn152.

**[0086]** It will be readily apparent to those of skill in the art that the numbering of amino acids in other

isoforms of JNK3 or JNK3 homologues may be different than that set forth for JNK3α1. Corresponding amino acids in other isoforms of JNK3 or JNK3 homologues are easily identified by visual inspection of the amino acid sequences or by using commercially available homology software programs.

**[0087]** Those of skill in the art understand that a set of structure coordinates for an enzyme or an enzyme-complex or a portion thereof, is a relative set of points that define a shape in three dimensions. Thus, it is possible that an entirely different set of coordinates could define a similar or identical shape. Moreover, slight variations in the individual coordinates will have little effect on overall shape. In terms of binding pockets, these variations would not be expected to significantly alter the nature of ligands that could associate with those pockets.

**[0088]** The variations in coordinates discussed above may be generated because of mathematical manipulations of the JNK3-inhibitor structure coordinates. For example, the structure coordinates set forth in Figure 1, 2 or 3 may undergo crystallographic permutations of the structure coordinates, fractionalization of the structure coordinates, integer additions or subtractions to sets of the structure coordinates, inversion of the structure coordinates or any combination of the above.

**[0089]** Alternatively, modifications in the crystal structure due to mutations, additions, substitutions, and/or deletions of amino acids, or other changes in any of the components that make up the crystal may also account for variations in structure coordinates. If such variations are within an acceptable standard error

as compared to the original coordinates, the resulting three-dimensional shape is considered to be the same. Thus, for example, a ligand that bound to the inhibitor-binding pocket of JNK3 would also be expected to bind to another binding pocket whose structure coordinates defined a shape that fell within the RMSD value.

**[0090]** Various computational analyses may be necessary to determine whether a binding pocket, motif, domain or portion thereof of a molecule or molecular complex is sufficiently similar to the binding pocket, motif, domain or portion thereof of JNK3. Such analyses may be carried out in well known software applications, such as ProFit [A. C.R. Martin, ProFit version 1.8, http://www.bioinf.org.uk/software], Swiss-Pdb Viewer [Guex et al., Electrophoresis, 18, pp. 2714-2723 (1997)], the Molecular Similarity application of QUANTA [Accelrys, San Diego, CA © 2001, 2002] and as described in the accompanying User's Guide, which are incorporated herein by reference.

**[0091]** The above programs permit comparisons between different structures, different conformations of the same structure, and different parts of the same structure. The procedure used in QUANTA and Swiss-Pdb Viewer to compare structures is divided into four steps: 1) load the structures to be compared; 2) define the atom equivalences in these structures; 3) perform a fitting operation on the structures; and 4) analyze the results. The procedure used in ProFit to compare structures includes the following steps: 1) load the structures to be compared; 2) specify selected residues of interest; 3) define the atom equivalences in the selected residues; 4) perform a

fitting operation on the selected residues; and

5) analyze the results.

[0092] Each structure is identified by a name. One structure is identified as the target (i.e., the fixed structure); all remaining structures are working structures (i.e., moving structures). Since atom equivalency within QUANTA is defined by user input, protein backbone atoms (N, Cα, C and O) or all protein atoms may be defined as equivalent atoms for JNK3 amino acids and corresponding amino acids in the structures being compared.

[0093] The corresponding amino acids may be identified by sequence alignment programs such as the "bestfit" program available from the Genetics Computer Group which uses the local homology algorithm described by Smith and Waterman in Advances in Applied Mathematics 2, 482 (1981), which is incorporated herein by reference. A suitable amino acid sequence alignment will require that the proteins being aligned share minimum percentage of identical amino acids. Generally, a first protein being aligned with a second protein should share in excess of about 35% identical amino acids [Hanks et al., Science, 241, 42 (1988); Hanks and Quinn, Methods in Enzymology, 200, 38 (1991)]. The identification of equivalent residues can also be assisted by secondary structure alignment, for example, aligning the α-helices, β-sheets in the structure. The program Swiss-Pdb viewer utilizes a best fit algorithm that is based on secondary sequence alignment.

[0094] When a rigid fitting method is used, the working structure is translated and rotated to obtain an optimum fit with the target structure. The fitting

operation uses an algorithm that computes the optimum translation and rotation to be applied to the moving structure, such that the root mean square difference of the fit over the specified pairs of equivalent atom is an absolute minimum. This number, given in angstroms, is reported by QUANTA.

**[0095]** The RMSD values between the backbone atoms of amino acid residues in the inhibitor-binding pocket of JNK3-inhibitor complexes (Figure 1 or 2) and those of the corresponding amino acid residues in the JNK3-AMP-PNP complex are illustrated in Table 2. Amino acid residues Lys93, Ile124, Leu126, Leu144, Met146 (SET1) and amino acid residues Ile70, Gly71, Ser72, Asn152, Cys154 and Gln155 (SET2) in the JNK3 inhibitor-binding pocket were used in the RMSD calculation. In addition, the RMSD values of the backbone atoms of the amino acid residues of JNK3-isoxazole1, JNK3-uracil1 and JNK3-thiazole1 compared to those of JNK3-AMP-PNP is 0.99 Å, 0.77 Å and 0.56 Å, respectively. The RMSD values of all atoms (including backbone and sidechain atoms) of the amino acid residues of JNK3-isoxazole1, JNK3-uracil1 and JNK3-thiazole1 compared to those of JNK3-AMP-PNP is 1.41 Å, 0.97 Å and 0.90 Å, respectively. The RMSD values are averages of individual RMSD values and were calculated by the program QUANTA.

Table 2

| Proteins | RMSD of SET1 amino acids (Å) | RMSD of SET2 amino acids (Å) |
|---|---|---|
| JNK3-isoxazole1 and JNK3-AMP-PNP [a] | 0.43 | |
| JNK3-uracil1 and JNK3-AMP-PNP | 0.37 | 1.15 |

a Xie et al., Structure, 6, pp. 983-991 (1998); PDB accession number 1JNK. The Protein Data Bank is an international repository for three dimensional structures and can be located at www.rcsb.org/pdb/.

**[0096]** For the purpose of this invention, any molecule, molecular complex, binding pocket, motif, domain thereof or portion thereof that is within a root mean square deviation for backbone atoms (N, Cα, C, O) when superimposed on the relevant backbone atoms described by structure coordinates listed in Figure 1, 2 or 3 are encompassed by this invention.

**[0097]** Therefore, one embodiment of this invention provides a crystalline molecule or molecular complex comprising a binding pocket defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues Ile70, Ser72, Val78, Ala91, Ile92, Lys93, Ile124, Leu126, Leu144, Val145, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Ser193, Val196 and Leu206 according to Figure 1, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues

of said molecule or molecular complex and said JNK3 amino acid residues is not greater than about 0.5, 0.3 or 0.2 Å.

**[0098]** The above set of amino acid residues may further comprise amino acid residues which correspond to JNK3 amino acid residues Pro69, Gly71, Gln75, Gly76, Ile77, Cys79, Ala80, Val90, Lys94, Leu95, Met115, Ser125, Leu127, Asn128, Tyr143, Leu153, Gln155, Pro192, Asn194, Ile195, Val197, Lys204 and Asp207 according to Figure 1, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said molecule or molecular complex and said JNK3 amino acid residues is not greater than about 0.8, 0.6, 0.4 or 0.2 Å.

**[0099]** One embodiment of this invention provides a crystalline molecule or molecular complex comprising a binding pocket defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues Ile70, Gln75, Val78, Ala91, Ile92, Lys93, Ile124, Leu126, Leu144, Val145, Met146, Leu148, Met149, Asp150, Ala151, Asn152, Ser193, Asn194, Val196 and Leu206 according to Figure 2, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said molecule or molecular complex and said JNK3 amino acid residues is not greater than about 0.6, 0.4 or 0.2 Å.

**[0100]** The above set of amino acid residues may further comprise amino acid residues which correspond to JNK3 amino acid residues Gly73, Gly76, Ile77, Cys79, Ala80, Val90, Lys94, Leu95, Arg107, Glu111, Met115, Ser125, Leu127, Asn128, Tyr143, Glu147, Leu153, Cys154, Gln155, Pro192, Ile195, Val197, Ile205 and Asp207 according to Figure 2, wherein the root mean square

deviation of the backbone atoms between said set of amino acid residues of said molecule or molecular complex and said JNK3 amino acid residues is not greater than about 0.7, 0.5, 0.3 or 0.2 Å.

**[0101]** One embodiment of this invention provides a crystalline molecule or molecular complex comprising a binding pocket defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues Ile70, Val78, Ala91, Lys93, Glu111, Ile124, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Gln155, Val196 and Leu206 according to Figure 3, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said molecule or molecular complex and said JNK3 amino acid residues is not greater than about 0.4, 0.3 or 0.2 Å.

**[0102]** The above set of amino acid residues may further comprise JNK3 amino acid residues Gly71, Ser72, Ile77, Cys79, Ala80, Val90, Ile92, Lys94, Leu95, Met115, Ser125, Leu144, Val145, Leu153, Cys154, Ser193, Asn194, Ile195, Val197, Lys198, Lys204, Ile205 and Asp207 according to Figure 3, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said molecule or molecular complex and said JNK3 amino acid residues is not greater than about 0.5, 0.3 or 0.2 Å.

**[0103]** In another embodiment of the invention provides a crystalline molecule or molecular complex comprising a binding pocket defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues Lys93 and Met146 according to Figure 1 or 2, wherein the root mean square deviation of the backbone atoms between said set

of amino acid residues of said molecule or molecular complex and said JNK3 amino acid residues is not greater than about 0.2 Å.

**[0104]** In another embodiment of the invention provides a crystalline molecule or molecular complex comprising a binding pocket defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues Lys93, Ile124, Leu126, Leu144 and Met146 according to Figure 1 or 2, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said molecule or molecular complex and said JNK3 amino acid residues is not greater than about 0.3 Å or 0.2 Å.

**[0105]** In another embodiment of the invention provides a crystalline molecule or molecular complex comprising a binding pocket defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues Ser193 and Asn152 according to Figure 2, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said molecule or molecular complex and said JNK3 amino acid residues is not greater than about 0.2 Å.

**[0106]** In another embodiment of the invention provides a crystalline molecule or molecular complex comprising a binding pocket defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues Ile70, Gly71, Ser72, Asn152, Cys154 and Gln155 according to Figure 3, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said molecule or molecular complex and said JNK3 amino acid

residues is not greater than about 1.0, 0.8, 0.6, 0.4 or 0.2 Å.

[0107] In another embodiment of the invention provides a crystalline molecule or molecular complex comprising a protein defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues set forth in Figure 1, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said protein and said JNK3 amino acid residues is not more than about 0.9, 0.7, 0.5 or 0.3 Å.

[0108] In another embodiment of the invention provides a crystalline molecule or molecular complex comprising a protein defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues set forth in Figure 2, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said protein and said JNK3 amino acid residues is not more than about 0.7, 0.5 or 0.3 Å.

[0109] In another embodiment of the invention provides a crystalline molecule or molecular complex comprising a protein defined by structure coordinates of a set of amino acid residues which correspond to JNK3 amino acid residues set forth in Figure 3, wherein the root mean square deviation of the backbone atoms between said set of amino acid residues of said protein and said JNK3 amino acid residues is not more than about 0.45, 0.3 or 0.2 Å.

[0110] In another embodiment of the invention provides a crystalline molecular complex comprising a protein defined by structure coordinates of a set of amino acid residues which are identical to JNK3 amino

acid residues set forth in Figure 1, wherein the root mean square deviation between all atoms of said set of amino acid residues of said protein and said JNK3 amino acid residues is not more than about 1.3, 1.1, 0.9, 0.7 or 0.5 Å.

**[0111]** In another embodiment of the invention provides a crystalline molecular complex comprising a protein defined by structure coordinates of a set of amino acid residues which are identical to JNK3 amino acid residues set forth in Figure 2, wherein the root mean square deviation between all atoms of said set of amino acid residues of said protein and said JNK3 amino acid residues is not more than about 0.9, 0.7, 0.5 or 0.3 Å.

**[0112]** In another embodiment of the invention provides a crystalline molecular complex comprising a protein defined by structure coordinates of a set of amino acid residues which are identical to JNK3 amino acid residues set forth in Figure 3, wherein the root mean square deviation between all atoms of said set of amino acid residues of said protein and said JNK3 amino acid residues is not more than about 0.8, 0.6, 0.4 or 0.2 Å.

**[0113]** In yet another embodiment of the invention provides a crystalline molecular complex comprising a protein kinase, wherein the protein kinase comprises a methionine residue that corresponds to Met146 of JNK3, wherein the $\chi 1$ angle is in the range of about -120° to -180° and 45° to 180°. In one embodiment, the protein kinase is a JNK3 protein or a JNK3 homologue. In one embodiment, the $\chi 1$ angle is in the range of -150° to -180°, the $\chi 2$ angle is in the range of -150° to -170°, and the $\chi 3$ angle is in the range of 95° to 135°. In

another embodiment, the χ1 angle is in the range of 60° to 80°, the χ2 angle is in the range of 155° to 175°, and the χ3 angle is in the range of -45° to -65°. In another embodiment, the χ1 angle is in the range of 135° to 155°, the χ2 angle is in the range of -115° to -135°, and the χ3 angle is in the range of -155° to -175°.

Computer Systems

**[0114]** According to another embodiment, this invention provides a machine-readable data storage medium, comprising a data storage material encoded with machine-readable data, wherein said data defines the above-mentioned molecules or molecular complexes. In one embodiment, the data defines the above-mentioned binding pockets by comprising the structure coordinates of said amino acid residues according to Figure 1, 2 or 3. To use the structure coordinates generated for JNK3 homologues thereof, or one of its binding pockets, it is at times necessary to convert them into a three-dimensional shape or to generate three-dimensional structural information from them. This is achieved through the use of commercially or publicly available software that is capable of generating a three-dimensional structure of molecules or portions thereof from a set of structure coordinates. In one embodiment, the three-dimensional structure may be displayed as a graphical representation.

**[0115]** Therefore, according to another embodiment, this invention provides a machine-readable data storage medium comprising a data storage material encoded with machine readable data. In one embodiment, a machine programmed with instructions for using said data, is

capable of generating a three-dimensional structure of any of the molecule or molecular complexes, or binding pockets thereof, that are described herein.

[0116] This invention also provides a computer comprising:

a) a machine-readable data storage medium comprising a data storage material encoded with machine-readable data, wherein the data defines any one of the above binding pockets or protein of the molecule or molecular complex;

b) a working memory for storing instructions for processing said machine-readable data;

c) a central processing unit (CPU) coupled to the working memory and to the machine-readable data storage medium for processing said machine readable data as well as an instruction or set of instructions for generating three-dimensional structural information of said binding pocket or protein; and

d) output hardware coupled to the CPU for outputting three-dimensional structural information of the binding pocket or protein, or information produced by using the three-dimensional structural information of said binding pocket or protein. The output hardware may include monitors, touchscreens, printers, facsimile machines, modems, disk drives, CD-ROMs, etc.

[0117] Three-dimensional data generation may be provided by an instruction or set of instructions such as a computer program or commands for generating a three-dimensional structure or graphical representation from structure coordinates, or by subtracting distances between atoms, calculating chemical energies for a JNK3

molecule or molecular complex or homologues thereof, or calculating or minimizing energies for an association of a JNK3 molecule or molecular complex or homologues thereof to a chemical entity. The graphical representation can be generated or displayed by commercially available software programs. Examples of software programs include but are not limited to QUANTA [Accelrys ©2001, 2002], O [Jones *et al.*, Acta Crystallogr. A47, pp. 110-119 (1991)] and RIBBONS [Carson, J. Appl. Crystallogr., 24, pp. 9589-961 (1991)], which are incorporated herein by reference. Certain software programs may imbue this representation with physico-chemical attributes which are known from the chemical composition of the molecule, such as residue charge, hydrophobicity, torsional and rotational degrees of freedom for the residue or segment, etc. Examples of software programs for calculating chemical energies are described in the Rational Drug Design section.

**[0118]** Information about said binding pocket or information produced by using said binding pocket can be outputted through display terminals, touchscreens, printers, modems, facsimile machines, CD-ROMs or disk drives. The information can be in graphical or alphanumeric form.

**[0119]** Figure 9 demonstrates one version of these embodiments. System 10 includes a computer 11 comprising a central processing unit ("CPU") 20, a working memory 22 which may be, e.g., RAM (random-access memory) or "core" memory, mass storage memory 24 (such as one or more disk drives or CD-ROM drives), one or more cathode-ray tube ("CRT") display terminals 26, one or more keyboards 28, one or more input lines 30,

and one or more output lines 40, all of which are interconnected by a conventional bi-directional system bus 50.

**[0120]** Input hardware 35, coupled to computer 11 by input lines 30, may be implemented in a variety of ways. Machine-readable data of this invention may be inputted via the use of a modem or modems 32 connected by a telephone line or dedicated data line 34. Alternatively or additionally, the input hardware 35 may comprise CD-ROM drives or disk drives 24. In conjunction with display terminal 26, keyboard 28 may also be used as an input device.

**[0121]** Output hardware 46, coupled to computer 11 by output lines 40, may similarly be implemented by conventional devices. By way of example, output hardware 46 may include CRT display terminal 26 for displaying a graphical representation of a binding pocket of this invention using a program such as QUANTA as described herein. Output hardware might also include a printer 42, so that hard copy output may be produced, or a disk drive 24, to store system output for later use. Output hardware may also include a display terminal, a CD or DVD recorder, ZIP™ or JAZ™ drive, or other machine-readable data storage device.

**[0122]** In operation, CPU 20 coordinates the use of the various input and output devices 35, 46, coordinates data accesses from mass storage 24 and accesses to and from working memory 22, and determines the sequence of data processing steps. A number of programs may be used to process the machine-readable data of this invention. Such programs are discussed in reference to the computational methods of drug discovery as described herein. Specific references to

components of the hardware system 10 are included as appropriate throughout the following description of the data storage medium.

**[0123]** Figure 10 shows a cross section of a magnetic data storage medium 100 which can be encoded with a machine-readable data that can be carried out by a system such as system 10 of Figure 9. Medium 100 can be a conventional floppy diskette or hard disk, having a suitable substrate 101, which may be conventional, and a suitable coating 102, which may be conventional, on one or both sides, containing magnetic domains (not visible) whose polarity or orientation can be altered magnetically. Medium 100 may also have an opening (not shown) for receiving the spindle of a disk drive or other data storage device 24.

**[0124]** The magnetic domains of coating 102 of medium 100 are polarized or oriented so as to encode in a manner that may be conventional, machine readable data such as that described herein, for execution by a system such as system 10 of Figure 9.

**[0125]** Figure 11 shows a cross section of an optically-readable data storage medium 110 which also can be encoded with such a machine-readable data, or set of instructions, which can be carried out by a system such as system 10 of Figure 9. Medium 110 can be a conventional compact disk read only memory (CD-ROM) or a rewritable medium such as a magneto-optical disk that is optically readable and magneto-optically writable. Medium 100 preferably has a suitable substrate 111, which may be conventional, and a suitable coating 112, which may be conventional, usually of one side of substrate 111.

[0126] In the case of CD-ROM, as is well known, coating 112 is reflective and is impressed with a plurality of pits 113 to encode the machine-readable data. The arrangement of pits is read by reflecting laser light off the surface of coating 112. A protective coating 114, which preferably is substantially transparent, is provided on top of coating 112.

[0127] In the case of a magneto-optical disk, as is well known, coating 112 has no pits 113, but has a plurality of magnetic domains whose polarity or orientation can be changed magnetically when heated above a certain temperature, as by a laser (not shown). The orientation of the domains can be read by measuring the polarization of laser light reflected from coating 112. The arrangement of the domains encodes the data as described above.

[0128] In one embodiment, the structure coordinates of said molecules or molecular complexes are produced by homology modeling of at least a portion of the structure coordinates of Figure 1, 2 or 3. Homology modeling can be used to generate structural models of JNK3 homologues or other homologous proteins based on the known structure of JNK3. This can be achieved by performing one or more of the following steps: performing sequence alignment between the amino acid sequence of an unknown molecule against the amino acid of JNK3; identifying conserved and variable regions by sequence or structure; generating structure co-ordinates for structurally conserved residues of the unknown structure from those of JNK3; generating conformations for the structurally variable residues in the unknown structure; replacing the non-conserved residues of JNK3

with residues in the unknown structure; building side chain conformations; and refining and/or evaluating the unknown structure.

[0129] For example, since the protein sequence of the catalytic domains of JNK3 and JNK1 or JNK2 can be aligned relative to each other, it is possible to construct models of the structures of JNK1 or JNK2, particularly in the regions of the active site, using the JNK3 structure. Software programs that are useful in homology modeling include XALIGN [Wishart, D. S. et al., Comput. Appl. Biosci., 10, pp. 687-88 (1994)] and CLUSTAL W Alignment Tool [Higgins D. G. et al., Methods Enzymol, 266, pp. 383-402 (1996)]. See also, U.S. Patent No. 5,884,230. These references are incorporated herein by reference.

[0130] To perform the sequence alignment, programs such as the "bestfit" program available from the Genetics Computer Group [Waterman in Advances in Applied Mathematics 2, 482 (1981), which is incorporated herein by reference] and CLUSTAL W Alignment Tool [Higgins D. G. et al., Methods Enzymol, 266, pp. 383-402 (1996), which is incorporated by reference] can be used. To model the amino acid side chains of JNK1 or JNK2, the amino acid residues in JNK3 can be replaced, using a computer graphics program such as "O" [Jones et al, (1991) Acta Cryst. Sect. A, 47: 110-119], by those of the homologous protein, where they differ. The same orientation or a different orientation of the amino acid can be used. Insertions and deletions of amino acid residues may be necessary where gaps occur in the sequence alignment. However, certain portions of the active site of JNK3 and its homologues are highly conserved with essentially no insertions and deletions.

[0131] Homology modeling can be performed using, for example, the computer programs SWISS-MODEL available through Glaxo Wellcome Experimental Research in Geneva, Switzerland; WHATIF available on EMBL servers; Schnare et al., J. Mol. Biol, 256: 701-719 (1996); Blundell et al., Nature 326: 347-352 (1987); Fetrow and Bryant, Bio/Technology 11:479-484 (1993); Greer, Methods in Enzymology 202: 239-252 (1991); and Johnson et al, Crit. Rev. Biochem. Mol Biol. 29:1-68 (1994). An example of homology modeling can be found, for example, in Szklarz G.D., Life Sci. 61: 2507-2520 (1997). These references are incorporated herein by reference.

[0132] Thus, in accordance with the present invention, data capable of generating the three dimensional structure of the above molecules or molecular complexes, or binding pockets thereof, can be stored in a machine-readable storage medium, which is capable of displaying three-dimensional structural information or a graphical three-dimensional representation of the structure.

Rational Drug Design

[0133] The JNK3 structure coordinates or the three-dimensional graphical representation generated from these coordinates may be used in conjunction with a computer for a variety of purposes, including drug discovery.

[0134] For example, the structure encoded by the data may be computationally evaluated for its ability to associate with chemical entities. Chemical entities that associate with JNK3 may inhibit or activate JNK3 or its homologues, and are potential drug candidates. Alternatively, the structure encoded by the data may be

displayed in a graphical three-dimensional representation on a computer screen. This allows visual inspection of the structure, as well as visual inspection of the structure's association with chemical entities.

**[0135]** Thus, according to another embodiment, the invention provides a method for designing, selecting and/or optimizing a chemical entity that binds to the molecule or molecular complex comprising the steps of:

(a) providing the structure coordinates of said molecule or molecular complex on a computer comprising the means for generating three-dimensional structural information from said structure coordinates; and

(b) designing, selecting and/or optimizing said chemical entity by employing means for performing a fitting operation between said chemical entity and said three-dimensional structural information of said molecule or molecular complex.

**[0136]** Three-dimensional structural information in step (a) may be generated by instructions such as a computer program or commands that can generate a three-dimensional structure or graphical representation; subtract distances between atoms; calculate chemical energies for a JNK3 molecule, molecular complex or homologues thereof; or calculate or minimize energies of an association of JNK3 molecule, molecular complex or homologues thereof to a chemical entity. These types of computer programs are known in the art. The graphical representation can be generated or displayed by commercially available software programs. Examples of software programs include but are not limited to

QUANTA [Accelrys ©2001, 2002], O [Jones *et al.*, Acta Crystallogr. A47, pp. 110-119 (1991)] and RIBBONS [Carson, J. Appl. Crystallogr., 24, pp. 9589-961 (1991)], which are incorporated herein by reference. Certain software programs may imbue this representation with physico-chemical attributes which are known from the chemical composition of the molecule, such as residue charge, hydrophobicity, torsional and rotational degrees of freedom for the residue or segment, etc. Examples of software programs for calculating chemical energies are described below.

**[0137]** Another embodiment of the invention provides a method for evaluating the potential of a chemical entity to associate with the molecule or molecular complex as described previously.

**[0138]** This method comprises the steps of: a) employing computational means to perform a fitting operation between the chemical entity and the molecule or molecular complex described before; b) analyzing the results of said fitting operation to quantify the association between the chemical entity and the molecule or molecular complex; and, optionally, c) outputting said quantified association to a suitable output hardware, such as a CRT display terminal, a CD or DVD recorder, ZIP™ or JAZ™ drive, a disk drive, or other machine-readable data storage device, as described previously. The method may further comprise generating a three-dimensional structure, graphical representation thereof, or both, of the molecule or molecular complex prior to step a). In one embodiment, the method is for evaluating the ability of a chemical entity to associate with the binding pocket of a molecule or molecular complex.

[0139] In another embodiment, the method comprises the steps of:

a) constructing a computer model of a binding pocket of the molecule or molecular complex;

b) selecting a chemical entity to be evaluated by a method selected from the group consisting of assembling said chemical entity; selecting a chemical entity from a small molecule database; de novo ligand design of said chemical entity; and modifying a known agonist or inhibitor, or a portion thereof, of a JNK3 protein or homologue thereof;

c) employing computational means to perform a fitting program operation between computer models of said chemical entity to be evaluated and said binding pocket in order to provide an energy-minimized configuration of said chemical entity in the binding pocket; and

d) evaluating the results of said fitting operation to quantify the association between said chemical entity and the binding pocket model, whereby evaluating the ability of said chemical entity to associate with said binding pocket.

[0140] In another embodiment, the invention provides a method of using a computer for evaluating the ability of a chemical entity to associate with the molecule or molecular complex, wherein said computer comprises a machine-readable data storage medium comprising a data storage material encoded with said structure coordinates defining said binding pocket and means for generating a three-dimensional graphical representation

of the binding pocket, and wherein said method comprises the steps of:

(a) positioning a first chemical entity within all or part of said binding pocket using a graphical three-dimensional representation of the structure of the chemical entity and the binding pocket;

(b) performing a fitting operation between said chemical entity and said binding pocket by employing computational means;

(c) analyzing the results of said fitting operation to quantitate the association between said chemical entity and all or part of the binding pocket; and

(d) outputting said quantitated association to a suitable output hardware.

**[0141]** The above method may further comprise the steps of:

(e) repeating steps (a) through (d) with a second chemical entity; and

(f) selecting at least one of said first or second chemical entity that associates with said all or part of said binding pocket based on said quantitated association of said first or second chemical entity.

**[0142]** Alternatively, the structure coordinates of the JNK3 binding pockets may be utilized in a method for identifying an agonist or antagonist of a molecule comprising a binding pocket of JNK3. This method comprises the steps of:

a) using a three-dimensional structure of the molecule or molecular complex to design, select or optimize a chemical entity;

b) contacting the chemical entity with the molecule or molecular complex;

c) monitoring the catalytic activity of the molecule or molecular complex; and

d) classifying the chemical entity as an agonist or antagonist based on the effect of the chemical entity on the catalytic activity of the molecule or molecular complex.

**[0143]** In one embodiment, step a) is performed using a graphical representation of the binding pocket or portion thereof of the molecule or molecular complex.

**[0144]** In another embodiment, the method comprises the steps of:

a) constructing a computer model of a binding pocket of the molecule or molecular complex;

b) selecting a chemical entity to be evaluated by a method selected from the group consisting of assembling said chemical entity; selecting a chemical entity from a small molecule database; de novo ligand design of said chemical entity; and modifying a known agonist or inhibitor, or a portion thereof, of a JNK3 protein or homologue thereof;

c) employing computational means to perform a fitting program operation between computer models of said chemical entity to be evaluated and said binding

pocket in order to provide an energy-minimized configuration of said chemical entity in the binding pocket; and

d) evaluating the results of said fitting operation to quantify the association between said chemical entity and the binding pocket model, whereby evaluating the ability of said chemical entity to associate with said binding pocket;

e) synthesizing said chemical entity; and

f) contacting said chemical entity with said molecule or molecular complex to determine the ability of said compound to activate or inhibit said molecule.

**[0145]** For the first time, the present invention permits the use of molecular design techniques to identify, select and design chemical entities, including inhibitory compounds, capable of binding to JNK3 or JNK3-like binding pockets, motifs and domains.

**[0146]** Applicants' elucidation of binding pockets on JNK3 provides the necessary information for designing new chemical entities and compounds that may interact with JNK3 or JNK3-like ATP-binding pockets, in whole or in part. Due to the homology in the kinase core between JNK3, JNK1 and JNK2, compounds that inhibit JNK3 are also expected to inhibit JNK1 and JNK2, especially those compounds that bind the ATP-binding pocket.

**[0147]** Throughout this section, discussions about the ability of a chemical entity to bind to, associate with or inhibit JNK3 binding pockets refers to features of the entity alone. Assays to determine if a compound

binds to JNK3 are well known in the art and are exemplified below.

[0148] The design of chemical entities that bind to or inhibit JNK3 binding pockets according to this invention generally involves consideration of two factors. First, the entity must be capable of physically and structurally associating with parts or all of the JNK3 binding pockets. Non-covalent molecular interactions important in this association include hydrogen bonding, van der Waals interactions, hydrophobic interactions and electrostatic interactions.

[0149] Second, the entity must be able to assume a conformation that allows it to associate with the JNK3 binding pockets directly. Although certain portions of the entity will not directly participate in these associations, those portions of the entity may still influence the overall conformation of the molecule. This, in turn, may have a significant impact on potency. Such conformational requirements include the overall three-dimensional structure and orientation of the chemical entity in relation to all or a portion of the binding pocket, or the spacing between functional groups of an entity comprising several chemical entities that directly interact with the JNK3 or JNK3-like binding pockets.

[0150] The potential inhibitory or binding effect of a compound on JNK3 binding pockets may be analyzed prior to its actual synthesis and testing by the use of computer modeling techniques. If the theoretical structure of the given compound suggests insufficient interaction and association between it and the JNK3 binding pockets, testing of the entity is obviated.

However, if computer modeling indicates a strong interaction, the compound may then be synthesized and tested for its ability to bind to a JNK3 binding pocket. This may be achieved by testing the ability of the compound to inhibit JNK3 using the assays described in Example 6. In this manner, synthesis of inoperative compounds may be avoided.

**[0151]** A potential inhibitor of a JNK3 binding pocket may be computationally evaluated by means of a series of steps in which chemical entities or fragments are screened and selected for their ability to associate with the JNK3 binding pockets.

**[0152]** One skilled in the art may use one of several methods to screen chemical entities or fragments for their ability to associate with a JNK3 binding pocket. This process may begin by visual inspection of, for example, a JNK3 binding pocket on the computer screen based on the JNK3 structure coordinates in Figure 1, 2 or 3 or other coordinates which define a similar shape generated from the machine-readable storage medium. Selected fragments or chemical entities may then be positioned in a variety of orientations, or docked, within that binding pocket as defined *supra*. Docking may be accomplished using software such as QUANTA and Sybyl, followed by energy minimization and molecular dynamics with standard molecular mechanics force fields, such as CHARMM and AMBER.

**[0153]** Specialized computer programs may also assist in the process of selecting fragments or chemical entities. These include:

1. GRID [P. J. Goodford, "A Computational Procedure for Determining Energetically Favorable

Binding Sites on Biologically Important Macromolecules", J. Med. Chem., 28, pp. 849-857 (1985)]. GRID is available from Oxford University, Oxford, UK.

2. MCSS [A. Miranker *et al.*, "Functionality Maps of Binding Sites: A Multiple Copy Simultaneous Search Method." Proteins: Structure, Function and Genetics, 11, pp. 29-34 (1991)]. MCSS is available from Molecular Simulations, San Diego, CA.

3. AUTODOCK [D. S. Goodsell *et al.*, "Automated Docking of Substrates to Proteins by Simulated Annealing", Proteins: Structure, Function, and Genetics, 8, pp. 195-202 (1990)]. AUTODOCK is available from Scripps Research Institute, La Jolla, CA.

4. DOCK [I. D. Kuntz *et al.*, "A Geometric Approach to Macromolecule-Ligand Interactions", J. Mol. Biol., 161, pp. 269-288 (1982)]. DOCK is available from University of California, San Francisco, CA.

**[0154]** Once suitable fragments have been selected, they can be assembled into a single compound or complex of compounds. Assembly may be preceded by visual inspection of the relationship of the fragments to each other on the three-dimensional image displayed on a computer screen in relation to the structure coordinates of JNK3. This would be followed by manual model building using software such as QUANTA or Sybyl [Tripos Associates, St. Louis, MO].

**[0155]** Useful programs to aid one of skill in the art in connecting the individual chemical entities or fragments include:

1. CAVEAT [P. A. Bartlett *et al.*, "CAVEAT: A Program to Facilitate the Structure-Derived Design of Biologically Active Molecules", in Molecular Recognition in Chemical and Biological Problems", Special Pub., Royal Chem. Soc., 78, pp. 182-196 (1989); G. Lauri and P. A. Bartlett, "CAVEAT: a Program to Facilitate the Design of Organic Molecules", J. Comput. Aided Mol. Des. , 8, pp. 51-66 (1994)]. CAVEAT is available from the University of California, Berkeley, CA.

2. 3D Database systems such as ISIS (MDL Information Systems, San Leandro, CA). This area is reviewed in Y. C. Martin, "3D Database Searching in Drug Design", J. Med. Chem., 35, pp. 2145-2154 (1992).

3. HOOK [M. B. Eisen *et al.*, "HOOK: A Program for Finding Novel Molecular Architectures that Satisfy the Chemical and Steric Requirements of a Macromolecule Binding Site", Proteins: Struct., Funct., Genet., 19, pp. 199-221 (1994)]. HOOK is available from Molecular Simulations, San Diego, CA.

**[0156]** Instead of proceeding to build an inhibitor of a JNK3 binding pocket in a step-wise fashion one fragment or chemical entity at a time as described above, inhibitory or other JNK3 binding compounds may be designed as a whole or "de novo" using either an empty binding pocket or optionally including some portion(s) of a known inhibitor(s). There are many de novo ligand design methods including:

1. LUDI [H.-J. Bohm, "The Computer Program LUDI: A New Method for the De Novo Design of Enzyme Inhibitors", J. Comp. Aid. Molec. Design, 6, pp. 61-78

(1992)]. LUDI is available from Molecular Simulations Incorporated, San Diego, CA.

2. LEGEND [Y. Nishibata *et al.*, Tetrahedron, 47, p. 8985 (1991)]. LEGEND is available from Molecular Simulations Incorporated, San Diego, CA.

3. LeapFrog [available from Tripos Associates, St. Louis, MO].

4. SPROUT [V. Gillet *et al.*, "SPROUT: A Program for Structure Generation)", J. Comput. Aided Mol. Design, 7, pp. 127-153 (1993)]. SPROUT is available from the University of Leeds, UK.

**[0157]** Other molecular modeling techniques may also be employed in accordance with this invention [see, e.g., N. C. Cohen *et al.*, "Molecular Modeling Software and Methods for Medicinal Chemistry, J. Med. Chem., 33, pp. 883-894 (1990); see also, M. A. Navia and M. A. Murcko, "The Use of Structural Information in Drug Design", Current Opinions in Structural Biology, 2, pp. 202-210 (1992); L. M. Balbes *et al.*, "A Perspective of Modern Methods in Computer-Aided Drug Design", Reviews in Computational Chemistry, Vol. 5, K. B. Lipkowitz and D. B. Boyd, Eds., VCH, New York, pp. 337-380 (1994); see also, W. C. Guida, "Software For Structure-Based Drug Design", Curr. Opin. Struct. Biology, 4, pp. 777-781 (1994)].

**[0158]** Once a chemical entity has been designed or selected by the above methods, the efficiency with which that chemical entity may bind to a JNK3 binding pocket may be tested and optimized by computational evaluation. For example, an effective JNK3 binding pocket inhibitor must preferably demonstrate a

relatively small difference in energy between its bound and free states (i.e., a small deformation energy of binding). Thus, the most efficient JNK3 binding pocket inhibitors should preferably be designed with a deformation energy of binding of not greater than about 10 kcal/mole, more preferably, not greater than 7 kcal/mole. JNK3 binding pocket inhibitors may interact with the binding pocket in more than one conformation that is similar in overall binding energy. In those cases, the deformation energy of binding is taken to be the difference between the energy of the free entity and the average energy of the conformations observed when the inhibitor binds to the protein.

**[0159]** An entity designed or selected as binding to a JNK3 binding pocket may be further computationally optimized so that in its bound state it would preferably lack repulsive electrostatic interaction with the target enzyme and with the surrounding water molecules. Such non-complementary electrostatic interactions include repulsive charge-charge, dipole-dipole and charge-dipole interactions.

**[0160]** Specific computer software is available in the art to evaluate compound deformation energy and electrostatic interactions. Examples of programs designed for such uses include: Gaussian 94, revision C [M. J. Frisch, Gaussian, Inc., Pittsburgh, PA ©1995]; AMBER, version 4.1 [P. A. Kollman, University of California at San Francisco, ©1995]; QUANTA/CHARMM [Accelrys, San Diego, CA ©2001, 2002]; Insight II/Discover [Accelrys, San Diego, CA ©2001, 2002]; DelPhi [Accelrys, San Diego, CA ©2001, 2002]; and AMSOL [Quantum Chemistry Program Exchange, Indiana University]. These programs may be implemented, for

instance, using a Silicon Graphics workstation such as an Indigo2 with "IMPACT" graphics. Other hardware systems and software packages will be known to those skilled in the art.

**[0161]** Another approach enabled by this invention, is the computational screening of small molecule databases for chemical entities that can bind in whole, or in part, to a JNK3 binding pocket. In this screening, the quality of fit of such entities to the binding pocket may be judged either by shape complementarity or by estimated interaction energy [E. C. Meng *et al.*, J. Comp. Chem., 13, pp. 505-524 (1992)].

**[0162]** Another particularly useful drug design technique enabled by this invention is iterative drug design. Iterative drug design is a method for optimizing associations between a protein and a compound by determining and evaluating the three-dimensional structures of successive sets of protein/compound complexes.

**[0163]** In iterative drug design, crystals of a series of protein or protein complexes are obtained and then the three-dimensional structures of each crystal is solved. Such an approach provides insight into the association between the proteins and compounds of each complex. This is accomplished by selecting compounds with inhibitory activity, obtaining crystals of this new protein/compound complex, solving the three-dimensional structure of the complex, and comparing the associations between the new protein/compound complex and previously solved protein/compound complexes. By observing how changes in the compound affected the

protein/compound associations, these associations may be optimized.

**[0164]** In some cases, iterative drug design is carried out by forming successive protein-compound complexes and then crystallizing each new complex. Alternatively, a pre-formed protein crystal is soaked in the presence of an inhibitor, thereby forming a protein/compound complex and obviating the need to crystallize each individual protein/compound complex.

Structure Determination of Other Molecules

**[0165]** The structure coordinates set forth in Figure 1, 2 or 3 can also be used to aid in obtaining structural information about another crystallized molecule or molecular complex. This may be achieved by any of a number of well-known techniques, including molecular replacement.

**[0166]** According to an alternate embodiment, the machine-readable data storage medium comprises a data storage material encoded with a first set of machine readable data which comprises the Fourier transform of at least a portion of the structure coordinates set forth in Figure 1, 2 or 3 or homology model thereof, and which, when using a machine programmed with instructions for using said data, can be combined with a second set of machine readable data comprising the X-ray diffraction pattern of a molecule or molecular complex to determine at least a portion of the structure coordinates corresponding to the second set of machine readable data.

**[0167]** In another embodiment, the invention provides a computer for determining at least a portion of the structure coordinates corresponding to X-ray

diffraction data obtained from a molecule or molecular complex, wherein said computer comprises:

a) a machine-readable data storage medium comprising a data storage material encoded with machine-readable data, wherein said data comprises at least a portion of the structural coordinates of JNK3 according to Figure 1, 2 or 3 or homology model thereof;

b) a machine-readable data storage medium comprising a data storage material encoded with machine-readable data, wherein said data comprises X-ray diffraction data obtained from said molecule or molecular complex; and

c) instructions for performing a Fourier transform of the machine readable data of (a) and for processing said machine readable data of (b) into structure coordinates.

**[0168]** For example, the Fourier transform of at least a portion of the structure coordinates set forth in Figure 1, 2 or 3 or homology thereof may be used to determine at least a portion of the structure coordinates of JNK3 homologues or other isoforms of JNK3.

**[0169]** Therefore, in another embodiment this invention provides a method of utilizing molecular replacement to obtain structural information about a molecule or molecular complex whose structure is unknown comprising the steps of:

a) crystallizing said molecule or molecular complex of unknown structure;

b) generating an X-ray diffraction pattern from said crystallized molecule or molecular complex; and

c) applying at least a portion of the structure coordinates set forth in Figure 1, 2 or 3 or homology model thereof to the X-ray diffraction pattern to generate a three-dimensional electron density map of the molecule or molecular complex whose structure is unknown; and

d) generating a structural model of the molecule or molecular complex from the three-dimensional electron density map.

**[0170]** In one embodiment, the method is performed using a computer. In another embodiment, the molecule is selected from the group consisting of JNK3 and JNK3 homologues. In another embodiment, the molecule is a JNK3 molecular complex or homologue thereof.

**[0171]** By using molecular replacement, all or part of the structure coordinates of the JNK3 as provided by this invention (and set forth in Figure 1, 2 or 3) can be used to determine the structure of a crystallized molecule or molecular complex whose structure is unknown more quickly and efficiently than attempting to determine such information *ab initio*.

**[0172]** Molecular replacement provides an accurate estimation of the phases for an unknown structure. Phases are a factor in equations used to solve crystal structures that can not be determined directly. Obtaining accurate values for the phases, by methods other than molecular replacement, is a time-consuming process that involves iterative cycles of approximations and refinements and greatly hinders the

solution of crystal structures. However, when the crystal structure of a protein containing at least a homologous portion has been solved, the phases from the known structure provide a satisfactory estimate of the phases for the unknown structure.

**[0173]** Thus, this method involves generating a preliminary model of a molecule or molecular complex whose structure coordinates are unknown, by orienting and positioning the relevant portion of the JNK3 according to Figure 1, 2 or 3 or homology model thereof within the unit cell of the crystal of the unknown molecule or molecular complex so as best to account for the observed X-ray diffraction pattern of the crystal of the molecule or molecular complex whose structure is unknown. Phases can then be calculated from this model and combined with the observed X-ray diffraction pattern amplitudes to generate an electron density map of the structure whose coordinates are unknown. This, in turn, can be subjected to any well-known model building and structure refinement techniques to provide a final, accurate structure of the unknown crystallized molecule or molecular complex [E. Lattman, "Use of the Rotation and Translation Functions", in Meth. Enzymol., 115, pp. 55-77 (1985); M. G. Rossmann, ed., "The Molecular Replacement Method", Int. Sci. Rev. Ser., No. 13, Gordon & Breach, New York (1972)].

**[0174]** The structure of any portion of any crystallized molecule or molecular complex that is sufficiently homologous to any portion of the JNK3 can be resolved by this method.

**[0175]** In a preferred embodiment, the method of molecular replacement is utilized to obtain structural information about a JNK3 homologue. The structure

coordinates of JNK3 as provided by this invention are particularly useful in solving the structure of JNK3 complexes that are bound by ligands, substrates and inhibitors.

**[0176]** Furthermore, the structure coordinates of JNK3 as provided by this invention are useful in solving the structure of JNK3 proteins that have amino acid substitutions, additions and/or deletions (referred to collectively as "JNK3 mutants", as compared to naturally occurring JNK3). These JNK3 mutants may optionally be crystallized in co-complex with a chemical entity, such an inhibitor or a suicide substrate. The crystal structures of a series of such complexes may then be solved by molecular replacement and compared with that of wild-type JNK3. Potential sites for modification within the various binding pockets of the enzyme may thus be identified. This information provides an additional tool for determining the most efficient binding interactions, for example, increased hydrophobic interactions, between JNK3 and a chemical entity or compound.

**[0177]** The structure coordinates are also particularly useful in solving the structure of crystals of JNK3 or JNK3 homologues complexed with a variety of chemical entities. This approach enables the determination of the optimal sites for interaction between chemical entities, including candidate JNK3 inhibitors. For example, high resolution X-ray diffraction data collected from crystals exposed to different types of solvent allows the determination of where each type of solvent molecule resides. Small molecules that bind tightly to those sites can then be

designed and synthesized and tested for their JNK3 inhibition activity.

**[0178]** All of the complexes referred to above may be studied using well-known X-ray diffraction techniques and may be refined versus 1.2-3.4 Å resolution X-ray data to an R value of about 0.30 or less using computer software, such as X-PLOR [Yale University, ©1992, distributed by Molecular Simulations, Inc.; see, e.g., Blundell & Johnson, supra; Meth. Enzymol., vol. 114 & 115, H. W. Wyckoff *et al.*, eds., Academic Press (1985)] or CNS [Brunger et al., Acta Crystallogr. D. Biol. Crystallogr., 54, pp. 905-921, (1998)]. This information may thus be used to optimize known JNK3 inhibitors, and more importantly, to design new JNK3 inhibitors.

**[0179]** In order that this invention be more fully understood, the following examples are set forth. These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any way.

## Example 1

### Expression and purification of JNK3

**[0180]** A BLAST search of the EST database using the published JNK3α1 cDNA [S. Gupta et al. (1996)] as a query identified an EST clone (#632588) that contained the entire coding sequence for human JNK3α1. Polymerase chain reactions (PCR) using *pfu* polymerase (Strategene) were used to introduce restriction sites into the cDNA for cloning into the pET-15B expression vector at the NcoI and BamHI sites for expression of the protein in *E. coli*. Due to the poor solubility of

the expressed full length protein (Met 1-Gln 422), an N-terminally truncated protein starting at Ser residue at position 40 (Ser 40), corresponding to Ser 2 of JNK1 and JNK2 proteins [S. Gupta et al. (1996)], preceded by Met (initiation) and Gly residues, were produced. The Gly residue was added in order to introduce an NcoI site for cloning into the expression vector. Further, systematic C-terminal truncations were performed by PCR to identify a construct that gave rise to diffraction-quality crystals. This construct, which was prepared by PCR using deoxyoligonucleotides 5' GCTCTAGAGCTCCATGGGCAGCAAAAGCAAAGTTGACAA 3' (SEQ ID NO: 5) (forward primer with initiation codon underlined) and 5' TAGCGGATCCTCATTCTGAA TTCATTACTTCCTTGTA 3' (SEQ ID NO: 6) (reverse primer with stop codon underlined) as primers and confirmed by DNA sequencing, encodes amino acid residues Ser40-Glu402 of JNK3α1, preceded by Met and Gly residues, was used for structural studies described herein. Control experiments indicated that the truncated JNK3 protein has an equivalent kinase activity towards myelin basic protein when activated with an upstream kinase MKK7 *in vitro*.

**[0181]** *E.coli* strain BL21 (DE3) (Novagen) transformed with the JNK3 expression construct was grown at 30°C in shaker flasks into log phase (OD600 ~ 0.8) in LB supplemented with 100 μg/ml carbenicillin. IPTG was then added to a final concentration of 0.8 mM and the cells were harvested 2 hours later by centrifugation.

**[0182]** *E.* coli cell paste containing the truncated JNK3 protein was resuspended in 10 volumes/g lysis buffer [50 mM HEPES, pH 7.2, 10% glycerol (v/v), 100 mM NaCl, 2 mM dithiothreitol (DTT), 0.1mM PMSF, 2 μg/ml

Pepstatin, 1μg/ml each of E-64 and Leupeptin]. Cells were lysed on ice using a microfluidizer and centrifuged at 100,000 x *g* for 30 min at 4°C. The 100,000 x *g* supernatant was diluted 5 fold with Buffer A [20 mM HEPES, pH 7.0, 10% glycerol (v/v), 2 mM DTT) and applied to an SP-Sepharose (Pharmacia) cation-exchange column at 4°C. The column was washed with 5 column volumes of Buffer A, followed by 5 column volumes of Buffer A containing 50 mM NaCl. Bound protein was eluted with a 7.5 column volume linear gradient of 50-300 mM NaCl, and the truncated JNK3 protein was eluted between 150-200 mM NaCl. Eluted JNK3 protein from the SP-Sepharose column was dialyzed at ~ 1 mg/ml against Buffer B [25 mM HEPES, pH 7.0, containing 5% glycerol (v/v), 50 mM NaCl, 10 mM DTT] overnight at 4°C and centrifuged at 3,000 x *g*. The supernatant was concentrated by ultrafiltration (Centriprep-30, Amicon) to 10 mg/ml, centrifuged at 16,000 x *g* and stored at -70°C.

## Example 2

## Crystallization of JNK3

**[0183]** Full length human JNK3α1 has a 39-residue extension in the N-terminus when compared to JNK1, JNK2 and other MAP kinases [Fig. 4 and S. Gupta et al. (1996)]. The conserved MAP kinase homologous region of JNK3 without the first 39 residues was used for crystallographic studies. Initial crystallization trials yielded only small crystals that diffracted to 8 Å. Since residues at the C-terminus of Erk2 and p38 are disordered [F. Zhang et al., Nature, 367, pp. 704-11 (1994); K. P. Wilson et al., J. Biol. Chem., 271,

pp. 27696-700 (1996)], it suggests that the C-terminal portions of JNK3 might also be flexible and interfere with the formation of a well-ordered crystal lattice. By combining limited proteolysis and systematic truncation of the protein, an active truncated JNK3 was searched. This screening approach resulted in the growth of larger, well-ordered JNK3 crystals. These crystals were grown from the JNK3 protein lacking the N-terminal 39 and C-terminal 20 residues. The truncated enzyme (residues Ser40-Glu402) displays wild-type kinase activity when activated by MKK7 *in vitro*. All crystallographic studies were carried out using this form of the enzyme.

**[0184]** Solutions of JNK3-inhibitor complex for crystallization were prepared by incubating the JNK3 protein solution (concentration of 10 mg/ml) with 0.5-1 mM inhibitor for 1 hour at room temperature prior to crystallization. Crystals of JNK3-inhibitor complexes were grown at 20°C by the vapour diffusion hanging-drop method over a reservoir solution containing 18-20% (v/v) polyethylene glycol monomethyl ether (average Mr = 550), 10% (v/v) ethylene glycol, 20 mM $\beta$-Mercaptoethanol and 100 mM Hepes (pH 7.0). However, growth of the JNK3-inhibitor crystals required microseeding the freshly made JNK3-inhibitor complex solution with seed stocks prepared from the JNK3-MgAMP-PNP crystals [WO 9957253, incorporated herein by reference]. The seed stock was prepared by crushing JNK3-MgAMP-PNP crystals to obtain a micro-crystal seed stock. The JNK3-inhibitor crystals belong to the orthorhombic space group *P212121* (with one enzyme molecule per asymmetric unit). The solvent content of the crystal is 44%. Before data collection, crystals

were equilibrated in their reservoir solution for 2-5 minutes before flash-frozen in nitrogen gas for X-ray data collection at -170°C.

Example 3

X-Ray data collection and structure determination

**[0185]** X-ray data were measured on either Raxis IIC or Raxis IV image plate mounted on a Rigaku RU200 rotating-anode generator (Rigaku/MSC). The diffraction images were processed with the program DENZO and data scaled using SCALPACK [Z. Otwinowski, In "Data Collection and Processing", L. Sawyer, N. Isaacs and S.W. Bailey, eds., Warrington, U.K.: Science and Engineering Council/Daresbury Laboratory. pp. 55-62 (1993)]. The data processing statistics are summarized in Table 3.

**[0186]** The X-ray coordinates of JNK3 from the JNK3-MgAMP-PNP structure [PDB acession number 1JNK] were used as a starting model for refinement of the JNK3-inhibitor complexes. All thermal factors were set to 20.0 $Å^2$. After one round of rigid body and positional refinement, the sigmaA-weighted |Fo|-|Fc| difference electron density maps were calculated using the CNS software package [Brunger et al., Acta Crystallogr. D. Biol. Crystallogr., 54, pp. 905-921, (1998)], and the bound inhibitors were identified. The inhibitor molecules were built and fit to the electron density in the program QUANTA. Then, the JNK3-inhibitor complex models were subjected to torsional dynamics (T=1000 to 4000 °C). Subsequent cycles of model rebuilding, positional refinement, and thermal factor refinement, intercepted with torsional dynamics runs were performed

to obtain the final model. All refinement procedures were carried out using CNS and model building was performed by the program QUANTA. A summary of the diffraction data and refinement statistics is provided in Table 3. The backbone conformation of 85% of the residues is within the most favored regions of the Ramachandran plot, with none in the disallowed region, as defined in the program PROCHECK [R. A. Laskowski et al., J. Appl. Crystallog., 26, pp. 283-91 (1993)].

Table 3

| Complex | JNK3-isoxazole1 | JNK3-uracil1 | JNK3-thiazole1 |
|---|---|---|---|
| Data resolution (Å) | 2.4 | 2.2 | 2.9 |
| Cell parameters | a= 54.75 Å<br>b= 70.48 Å<br>c= 107.66 Å<br>$\alpha$= 90°<br>$\beta$= 90°<br>$\gamma$= 90° | a= 51.65 Å<br>b= 71.01 Å<br>c= 106.7 Å<br>$\alpha$= 90°<br>$\beta$= 90°<br>$\gamma$= 90° | a= 50.74 Å<br>b= 71.82 Å<br>c= 107.22 Å<br>$\alpha$= 90°<br>$\beta$= 90°<br>$\gamma$= 90° |
| $R_{cryst}$ | 23.2 | 24.1 | 21.8 |
| $R_{free}$† | 30.5 | 31.9 | 29.2 |

† $R_{free}$ was calculated for a randomly chosen 5% of reflections for JNK3-thiazole1, and a randomly chosen 10% reflections for both JNK3-isoxazole1 and JNK3-uracil1.

**[0187]** In the final models, disordered residues were not included in the model. Alanine or glycine residues

were used in the model if the side chains of certain residues could not be located in the electron density.

Example 4

Overall Structure

**[0188]** The crystal structure of JNK3-isoxazole1 includes unphosphorylated JNK3 (residues 44-72, 75-212, 225-372 and 382-400) and isoxazole1. Electron density for residues 40-43, 73-74, 213-224 and 401-402 are missing, and these amino acid residues are presumed disordered. The crystal structure of JNK3-uracil1 includes unphosphorylated JNK3 (residues 45-70, 73-211, 217-373 and 379-400) and uracil1. Electron density for residues 40-44, 71-72, 212-216, 374-378 and 401-402 are missing, and these amino acid residues are presumed disordered. The crystal structure of JNK3-thiazole1 includes unphosphorylated JNK3 (residues 45-72, 77-211, 217-221 and 224-400) and uracil1. Electron density for residues 40-44, 73-76, 212-216, 222-223, and 401-402 are missing, and these amino acid residues are presumed disordered.

**[0189]** The overall architecture of JNK3 is substantially the same as JNK3 in the JNK3-AMP-PNP complex [WO 9957253] and highly similar to that of Erk2 and p38. However, the conformation in the active site differs. The MAP kinase homologous region of JNK3 (Phe48-Glu397) is 45% identical in amino acid sequence to Erk2 and 51% to p38, whose structures have been reported (F. Zhang et al. (1994); K. P. Wilson et al. (1996); Fig 4). The N-terminal lobe (residues 45-149, and 379-400) of JNK3 contains mostly beta-strands, whereas the C-terminal lobe (residues 150-211, 217-374)

is predominantly alpha-helical. In JNK3, the MAP kinase insertion in the C-terminal domain is 12 residues longer compared to Erk2 and p38, resulting in the N-terminal extension of helix αH and an extra 310 helix, denoted 3/10(2)L14 between αH and α3L14. This 12-residue insertion is referred to as "the JNK insertion" since it is present in all c-Jun N-terminal kinases [S. Gupta et al., (1996)].

Active site

**[0190]** The active site is situated in a deep cleft between the two domains of JNK3. JNK isoform sequences show that the amino acid residues involved in ATP binding are conserved [Gupta et al., EMBO J. 15, pp. 2760-2770 (1996)]. The catalytic core of protein kinases contains a nucleotide binding sequence Gly-X-Gly-X-X-Gly-X-X that is referred to as "the glycine-rich phosphate anchor loop" due to its structural feature and role in the nucleotide binding [D. R. Knighton et al., Science, 253, pp. 407-13 (1991)]. The conserved Asp189 and Asp207, both are thought to be essential for protein kinase activity [C. S. Gibbs et al., J. Biol. Chem., 267, pp. 4806-10 (1992)].

Inhibitor-binding pocket

**[0191]** All three compounds bind to the active site of JNK3 (Fig. 8). However, the exact spaces they occupy differ from each other due to the differences in their scaffolds and substituents. The phenyl rings from both uracil1 and isoxazole1 are buried in a hydrophobic pocket formed mainly by Met146 and Lys93, leaving minimal space for additional substituents to the ring. This pocket is normally unavailable when no ligand is

bound in the active site or when the bulky group corresponding to the phenyl ring in uracil1 and isoxazole1 is not present in the bound ligand, such as with ATP, AMP-PNP or thiazole1.

**[0192]** As illustrated in Figure 6, the binding of uracil1 induces a flip in the orientation of Met146. In order to avoid the clash with the phenyl group of uracil1, the Met146 takes a different conformation to accommodate the inhibitor. Compared to the Met146 in the JNK3-AMP-PNP structure, when viewed along the Cα-Cβ bond, the Cγ of Met146 in JNK3-uracil1 and JNK3-isoxazole1 swing about 110° clockwise, and about 125° counter clockwise, respectively. This conformational change results in the movement of Ile124 and Ser125 in the inhibitor binding-pocket, which further affects the conformation of other surrounding residues Asn101 to Cys117. Helix C (Figure 6) undergoes a pivoting movement around its N-terminus, and the C-terminal portion of Helix C swings out about 3 Å as a result of the movement in Ile124. The movement of helix C results in the disruption of the salt bridge between Glu111 of Helix C and Lys93 of the active site. This salt bridge is required for ATP hydrolysis.

**[0193]** In the JNK3-isoxazole1 and JNK3-uracil1 complexes, the backbone atoms of Lys93, Ile124, Leu126, Leu144 and Met146 are altered compared to those in the JNK3-AMP-PNP crystal structure. Table 4 shows a comparison of the dihedral angles for those residues. As will be understood by those skilled in the art, the $\phi_n$ angle refers to the rotation around the bond between the alpha carbon and the nitrogen, and the $\Psi_n$ angle refers to the rotation around the bond between the carbonyl carbon and the alpha carbon. The subscript

"n" identifies the amino acid whose conformation is being described [for a general reference, see Blundell and Johnson, Protein Crystallography, Academic Press, London, 1976].

Table 4

| Protein/($\phi$, $\Psi$)° | Lys93 | Ile124 | Leu126 | Leu144 | Met146 |
|---|---|---|---|---|---|
| JNK3-AMP-PNP | -109, 129 | -43, 134 | -95, 130 | -99, 127 | -134, 171 |
| JNK3-isoxazole1 | -135, 130 | -62, 129 | -72, 120 | -122, 127 | -157, 174 |
| JNK3-uracil1 | -114, 129 | -58, 137 | -95, 136 | -110, 143 | -143, 162 |
| P38α-apo | -106, 125 | -59, 141 | -90, 120 | -122, 139 | -137, 174 |
| P38α-compound | -106, 130 | -64, 138 | -99, 122 | -119, 137 | -143, 169 |

The $\phi$, $\Psi$ angles of Met146 for the JNK3-thiazole1 complex are -124° and -171°, respectively.

**[0194]** In addition, the number of hydrogen bonds and contacts that these compounds make with JNK3 are also different from each other (Fig. 8). In the case of AMP-PNP, the adenine base of the nucleotide is bound in the back of the domain interface, forming two hydrogen bonds to the protein backbone atoms in the hinge region. The amino group (N6) forms a hydrogen bond to the backbone carbonyl of Glu147 ("H-bond I"), and N1 accepts a proton from the backbone amide of Met149 ("H-bond II"). As shown in Fig. 8, H-bond II is present in

all three JNK3-inhibitor complexes, with the one in JNK3 uracil1 complex mediated by a water molecule. H-bond I is only present in the JNK3-isoxazole1 complex, where the aromatic carbon from the pyrimidine ring donates a proton to the backbone carbonyl of Glu147. Additionally, both isoxazole1 and uracil1 form hydrogen bonds with the backbone carbonyl of Met149.

## Example 5

## The Use of JNK3-inhibitor Coordinates for Inhibitor Design

**[0195]** The coordinates of Figure 1, 2 and 3 were used to design compounds, including inhibitory compounds, that associate with JNK3 or homologues of JNK3. This process was aided by using a computer comprising a machine-readable data storage medium encoded with a set of machine-executable instructions, wherein the recorded instructions are capable of displaying a three-dimensional representation of the JNK3-inhibitor complex or a portion thereof.

**[0196]** Results from enzyme assays have shown that among the three compounds, thiazole1 is the poorest inhibitor of JNK3 (Ki=13 $\mu$M), while uracil1 and isoxazole1 are quite comparable to each other (~0.8 $\mu$M). Our structural studies showed that the main difference between thiazole1 and the other compounds is its lack of a bulky substituent accessing the hydrophobic pocket, indicating the importance of retaining such bulky substituents for achieving high potency. Furthermore, isoxazole1 is the smallest molecule among the three compounds. While isoxazole1

occupies the least space in the active site, it sits closest to the protein and thereby makes the greatest number of direct hydrogen bonds. Therefore, it may be most advantageous to select isoxazole1 as the lead molecule for optimization.

[0197] Structural comparison revealed that several subsites occupied by thiazole1 and uracil1 are not accessed by isoxazole1. Although thiazole1 and uracil1 make fewer direct contacts with the JNK3 protein (Fig. 8), the additional subsites they occupy may contribute to their binding affinities. This suggests that modifications of isoxazole1 to access these subsites might help to further improve its potency. The subsite identified in the JNK3-thiazole1 structure consists of residues Ile70, Gly71, Ser72, Asn152, Cys154 and Gln155. These residues were in direct contact with thiazole-2-yl-pyridine of thiazole1 and phenyl of uracil1 (Fig. 7B). This subsite is at the entrance of the active site and is partially exposed to the solvent, indicating that it might be possible to improve the potency as well as the solubility of isoxazole1 by accessing this structurally important subsite. When the acetaldehyde of isoxazole1 was replaced by a bulky group such as a phenyl group or a cyclohexyl group, the potency improved significantly. The subsite identified in the JNK3-uracil1 structure consists of residues Ser72, Ser193 and Asn152. These residues surround the pyrimidine-2,4-dione group of uracil1 and the ribose group of AMP-PNP (Fig. 7C), suggesting that additional substitutions at O1 of isoxazole1 and/or modification of the 5-methyl may be helpful for further improving the properties of isoxazole1-derived ligands.

[0198] The above example illustrates how structural information from different JNK3-inhibitor complexes can be used to design a better class of compounds.

Example 6

JNK3 Activity Inhibition Assay

A. JNK3 activation

[0199] Five mg of JNK3 was diluted to 0.5 mg/ml in 50 mM HEPES buffer, pH 7.5, containing 100 mM NaCl, 5 mM DTT, 20 mM $MgCl_2$, 1 mM ATP. GST-MKK7(DD) kinase (the upstream mutant form of one of the activating kinases of JNK3) was added at a molar ratio of 1 GST-MKK7:2.5 JNK3. After 30 min at 25°C the reaction mixture was concentrated 5-fold by ultrafiltration in a Centriprep-30 (Amicon, Beverly, MA), then diluted back up to 10 ml and an additional 1 mM ATP added. This procedure was repeated three times to remove ADP and replenish ATP. The final (third) addition of ATP was 5 mM and the mixture incubated overnight at 4°C.

[0200] The activated JNK3/GST-MKK7(DD) reaction mixture was exchanged into 50 mM HEPES buffer, pH 7.5, containing 5 mM DTT and 5% glycerol (w/v) by dialysis or ultrafiltration. The reaction mixture was adjusted to 1.1 M potassium phosphate, pH 7.5, and purified by hydrophobic interactions chromatography (at 25°C) using a Rainin Hydropore column. GST-MKK7 and unactivated JNK3 do not bind under these conditions and when a 1.1 to 0.05 M potassium phosphate gradient is developed over 60 min at a flow rate of 1 ml/min, doubly phosphorylated JNK3 is separated from singly phosphorylated JNK3.

[0201] Activated JNK3 (i.e. doubly phosphorylated) was stored at -70°C at 0.25-1 mg/ml.

B. JNK3 Inhibition Assay

[0202] To determine the $IC_{50}$ of the compound binding to JNK3, the kinase activity of JNK3 was monitored by a coupled enzyme assay. In this assay, for every molecule of ADP generated by the JNK3 kinase activity one molecule of NADH is converted to NAD which can be conveniently monitored as an absorbance decrease at 340 nm. The following are the final concentrations of various reagents used in the assay: 100 mM HEPES buffer, pH 7.6, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 30 μM ATP, 2 mM phosphoenolpyruvate, 2 μM pyruvate kinase, 2 μM lactate dehydrogenase, 200 μM NADH, 200 μM EGF receptor peptide KRELVEPLTPSGEAPNQALLR (SEQ ID NO: 7), and 10 nM activated JNK3. First, all of the above reagents with the exception of ATP were mixed and 175 μl aliquots were placed per well in a 96-well plate. A 5 μl DMSO solution of the compound was added to each well, mixed, and allowed to stand at 30°C for 10 minutes. Typically about 10 different concentrations of the compound were tested. The reactions were initiated with the addition of 20 μl of ATP solution. Absorbance change at 340 nm were monitored as a function of time. $IC_{50}$ was obtained by fitting the rates vs. compound concentration data to a simple competitive inhibition model.

[0203] While we have described a number of embodiments of this invention, it is apparent that our basic constructions may be altered to provide other embodiments that utilize the products, processes and methods of this invention. Therefore, it will be

appreciated that the scope of this invention is to be defined by the appended claims, rather than by the specific embodiments that have been presented by way of example.